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(71) Applicant *(for all designated States except US)*: **THE VICTORIA UNIVERSITY OF MANCHESTER** [GB/GB]; Oxford Road, Manchester M13 9PL (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DOUGLAS, Kenneth, Thomas** [GB/GB]; Higher Shore Cottage, Higher Shore Road, Littleborough OL15 9LW (GB). **BICHENKOVA, Elena, Vladimirovna** [LV/GB]; 2 Egerton Court, Upper Park Road, Manchester M14 5SL (GB). **SARDARIAN, Ali** [IR/GB]; Flat 210, Horniman House, 66 Grafton Street, Manchester M13 9NT (GB).

(74) Agent: **ATKINSON, Peter, Birch**; Marks & Clerk, Sussex House, 83-85 Mosley Street, Manchester M2 3LG (GB).

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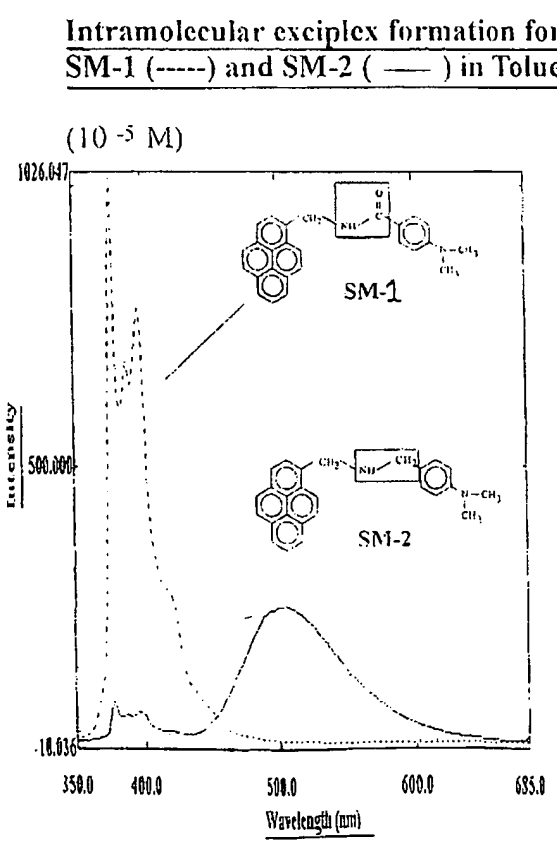
(54) Title: EXCIPLEXES



(57) Abstract: Compounds capable of forming an intramolecular exciplex on photoirradiation of the compound in water comprise two exciplex forming partners, one being a donor moiety and the other an acceptor moiety, each having at least one aromatic nucleus and being connected by a saturated aliphatic chain having the flexibility to allow said partners to come into exciplex forming relationship. The compounds may be used as labels for oligonucleotides. Certain of the compounds display pH sensitive emission.

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EXCIPLEXES

The present invention relates to exciplexes, i.e. heterocomplex analogues of excimers.

An excimer is a fluorescent complex formed when two identical complex forming partners (e.g. pyrene) are brought into the correct positional relationship to each other and photoirradiated. An exciplex (the heterocomplex analogue of an excimer) is formed on photoirradiation when (different) donor and acceptor species (e.g. pyrene and dimethyl aniline) come into the correct positional relationship to each other, the exciplex complex then dissociating with emission of fluorescence which is detectably different from that of either of the exciplex forming partners.

Exciplexes have the characteristics that is possible by altering the electron affinity and ionisation potential of the contributing partners to "tune" the emission wavelength of the complex including the wavelength and temporal characteristics, as may be used in time-resolved fluorescence. For example, an exciplex formed from N,N-diethylamine with chrysene emits at ca 420nm but one formed with N,N-diethylamine with perylene emits at ca 520nm

More particularly, the emission characteristics can be tuned in a predictable sense by the fine chemical structures of the partners, for example the emitted light frequency being linearly related to the difference in electron donor/electron acceptor strengths of the partners (see for example D.RehM, S. Naturforsch (1970) Vol 25a 1442-1447; J.B. Birks "Photophysics of Aromatic Molecules" published by Wiley Interscience, London.

Solvent polarity is crucial to the behaviour of exciplexes and *inter*molecular exciplexes do not emit usually in solvents as polar as acetonitrile[1-5]. In contrast, several *intra*molecular exciplexes exhibit exciplex emission in solvents up to the polarity of acetonitrile [6-15]. This behavioural shift has been ascribed to a change in structure for exciplexes going from compact in nonpolar solvents to loose in polar

solvents [13;14;16]. For strongly interacting exciplex partners, such as aromatic hydrocarbons with dialkylanilines (the most widely studied family, and certainly dominating the literature until very recently), the exciplex arises from a partial charge-transferred state, which is sufficiently stable in nonpolar solvents to fluoresce. Increased solvent polarity preferentially solvates and stabilises charge separation and at a dielectric constant of approximately 14 the pyrene:diethylaniline pair has an *exciplex absorption spectrum* identical with the ion pair, pyrene$^-$: $PhNEt_2^+$. Fluorescence quantum yields and lifetimes of exciplexes usually decrease with solvent polarity [3-5;11] the former more sensitively, but effects on lifetimes are more variable.

Intramolecular exciplexes have recently been discovered which emit in solvents as polar as DMSO, or even 20% aqueous CH_3CN [17]. Intermolecular exciplex luminescence in polar: nonpolar solvent mixtures (such as DMSO-benzene [18] or water-THF or water- dioxane [19] can be enhanced by magnetic fields. In none of these situations was use of a fully aqueous medium possible to observe exciplex emission even with the enhancement of magnetic field application. Literature data indicate that increasing water percentage in mixed solvents suppresses exciplex formation [12;17].

Emission of exciplexes in fully aqueous media is rarer and usually only occurs under special circumstances, for example in the special environments provided by some biomacromolecules, or in the presence of additives (such as cyclodextrins[20;21], cyclophanes [22] , polyanions [23]),. Exciplexes in water can be stabilised by polyanions (especially poly(vinyl sulphate), but also chondroitin sulfate C and heparin). The exciplex formed from excited 3,3'-diethylthiacyanine iodide (THIA) and cool acridine orange (AO) in the presence of chondroitin sulphate and other polyanions including DNA (emitting at 560nm) does not form in their absence and is destroyed by an increase in percent ethanol as co-solvent[23]. The enhancement of THIA fluorescence by the matrix depends also on structure of the polyanionic matrix (chondroitin sulphate C is about 4 times as effective chondroitin sulphate A). DNA enhances THIA fluorescence twice as strongly as chondroitin sulfate C (data quoted but unpublished). Heparin also facilitates exciplex formation in the aqueous environment

of THIA bound to a polymer. The synthetic polymer poly (vinylsulphate) quenches the monomer fluorescence of AO completely, but facilitates exciplex emission between THIA and AO (λ_{emit} = 560 nm). The authors provide arguments that the emissions detected are indeed exciplexes and not heteroaggregates of THIA and AO. In the absence of such additives, aqueous exciplex emission, if it exists, is too weak to have been detected to date.

Inorganic exciplexes behave differently from exciplexes formed from neutral organic molecules. For the $Ru(bpy)_3^{2+}$ and $Ru(phen)_3^{2+}/Ag^+$ systems exciplex emission spectra are much less red-shifted with increased [CH_3CN] in H_2O: CH_3CN mixtures relative to the spectrum in H_2O. This study showed that exciplex stabilisation by solvent polarity has the opposite tendency for neutral molecules compared to the exciplex from two ionic species of the same sign[24;25]. In spite of the enormous effect that addition of MeCN to H_2O has on the overall properties of the RuL_3^{2+}/Ag^+ exciplex system, these compositional changes have a remarkably small effect on the formation constants for the Ag^+ exciplexes (even though solvent changes affect photo-properties)[24].

The use of applied magnetic fields has permitted exciplex emission to be studied in water-solvent mixtures such as water-dioxan up to 20% water v/v. For a series of intramolecular exciplexes, exciplex fluorescence increased steeply as the magnetic field went from 0 to 1.0 Tesla (and then decreased gradually to 9 T (similarly the mean lifetimes)[18]. The exciplex luminescence increase found with increased magnetic fields is dependent on: (a) the particular exciplex partners, (b) the particular solvent mixtures (the increase is maximal at γ=15 for THF: MeOH and ~17 for dioxan: H_2O), (c) the magnetic field strength (plateau at approx 150 Gauss for 520 nm emission of pyrene: DMA and (d) the ionic strength[19].

Exciplexes of undefined structures have been reported for proteins and (oligo)nucleotides and nucleic acids with a number of ligands, which interact with some part(s) of the protein (such as tryptophan residues) or the nucleic acid/ nucleotide (usually base or base-pair(s)). In view of the complexity of these systems

and the lack of precision in structural definition currently available for the contributors to the exciplex partners, such systems have not permitted the impact of a fully aqueous environment on exciplex properties to be explored.

There is no report of intramolecular organic exciplex emission in fully aqueous media, and consequently no information of the influence on exciplex properties of media parameters, including ionic strength, temperature, pH, co-solvents, buffer materials, metal ions, detergents etc.

Cox et al [118] have observed a pH-dependent increase in an exciplex signal following an ionisation profile corresponding to a pH value of 8.9. This discovery had the following limitations: (1) it only occurred for 1-α-naphthyl-3-(dimethylamino)propane in the presence of high concentrations (0.01M) of β–cyclodextrin and the pH sensitivity was such that the maximal change in signal occurred between pH 8 and 10, outside the physiological range; (2) the binding strength of the complex formed between 1-α-naphthyl-3-(dimethylamino)propane and β–cyclodextrin, necessary for exciplex signal to be detectable, was weak and thus extremely high concentrations of β–cyclodextrin would have to be added (this would limit use in living tissue in view of the properties and toxicities of β–cyclodextrin).

According to a first aspect of the present invention there is provided a compound capable of forming an intramolecular exciplex on photoirradiation of the compound in water, said compound comprising two exciplex forming partners each having at least one aromatic nucleus and being connected by a saturated aliphatic chain having the flexibility to allow said partners to come into exciplex forming relationship.

The present invention thus provides the significant advance of compounds which are capable of forming intramolecular exciplexes in water. Such exciplex formation may be achieved in systems which are, in effect, 100% aqueous but formation is not limited to such systems and may also be achieved in liquids which

are partly aqueous and also in non-aqueous medium polarity liquids (e.g. THF) and low polarity liquids (e.g. toluene). The exciplex emission is achieved without the need for additives such as cyclodextrin or polyanionic compounds and without the requirement for application of the magnetic field.

The compounds of the invention allow the use of aqueous media to exploit properties of exciplexes that were previously restricted to non- or low-percentage aqueous environments such as lasers, dyes to act as fluorescent or circular dichroism detectors when the intra molecular exciplexes have been used to label other materials including proteins, nucleic acids and (oligo)nucleotides and their analogues such as PNAs, glycoproteins, solid materials including ceramics, transistors, semiconductors, insulators and glasses.

Moreover, as detailed more fully below, at least certain of the compounds in accordance with the invention provide pH sensitive exciplex emission in aqueous systems. Thus for example, there may be no exciplex emission below a certain pH but emission above that pH. Alternatively or additionally the emission characteristics may vary continually over a pH range. Thus for the compounds which demonstrate pH sensitive emission may be used as the basis for a molecular pH trigger or switch. More particularly, and by way of example only, certain compounds in accordance with the invention (e.g. **SM-2** – see Example 1 *infra*) have sensitivity to fluorescence of the exciplex at around pH 7.5 and may be used as the basis for analytical methods for *in vitro* systems or cell based systems. At low pH (e.g. pH 6) there is no detectable signal but changing the pH to 7.5 or higher gives exciplex emission, this the compound (or an analytical device based on the compound) could be used, without the need for additives such as cyclodextrins as was necessary in the work of Cox et al, to:

(a) register pH changes in an environment such as a living or fixed cell, or in a curvette or other container including cell sorter devices, and be used in assays to other determinations, or

(b) induce a signal by manipulation of the pH of the environment externally, such as by added acid, base or other change in conditions which leads to a pH change.

Thus according to a second aspect of the present invention there is provided a method of detection wherein there is used, as a label, a compound in accordance with the invention and said method comprises irradiating the sample under test with electromagnetic radiation capable of providing exciplex formation in said label (if present) and detecting for exciplex formation.

The sample which is irradiated preferable has an alkaline pH. The method may involve increasing the pH of the sample (e.g. from neutral to alkaline) to achieve exciplex formation.

The compounds in accordance with the invention comprise two exciplex forming partners each having at least one aromatic nucleus and being connected by a saturated aliphatic chain having a length and flexibility to allow the partners to come into exciplex forming relationship on irradiation with light of the appropriate wavelength. By the term "saturated aliphatic chain" we mean that "line" of atoms which links the two exciplex forming partners does not include an atom in that "line" which has move than one bond to another single atom. Thus, for example the carbon atom of a carbonyl group is regarded as being an unsaturated atom. The presence of unsaturated atoms in the aliphatic linker chain will restrict rotation (about the unsaturated bond) and act to prevent the (potential) exciplex partners coming into exciplex forming relationship.

It should be noted that the saturated aliphatic linker chain may itself be substituted (possibly with groups containing unsaturated atoms provided that such atoms are not in the "line" of atoms connecting the two exciplex forming partners), such substituted compounds are also to be regarded as being within the scope of the invention, the substitution may for example be with a functional group (e.g. an amino group) which permits covalent attachment of the compound (as a label) to a molecule

to or other entity to be labelled, e.g. a nucleic acid, (oligo)nucleotide or protein. Such labelled entities are also to be regarded as being within the scope of the invention.

Preferably the saturated aliphatic linker comprises 2 to 9 saturated atoms as the link between the two exciplex-forming partners. More preferably the linker comprises 2 to 4, and ideally 3, saturated atoms as the link. The saturated atoms if the linker may be carbon atoms (e.g. provided by methylene groups) or may be comprised partially or wholly of other atoms, e.g. nitrogen, sulfur or oxygen.

Preferred examples of linker include $-CH_2-CH_2-$, $CH_2-CH_2-CH_2$, and $-CH_2-NH-CH_2-$ in which the free terminal bonds are bonded to the exciplex forming partners.

The exciplex forming partners in compounds of the invention will comprise donor and acceptor moieties (each incorporating at least one aromatic nucleus) which may be selected (by way of example only) from residues of any of the following compounds, namely

(i) benzene, naphthalene, anthracene, phenanthrene, pyrene and chrysene, phenanthrene;

(ii) derivatives of residues of compounds identified under (i), particularly (but not exclusively) amino or substituted amino derivatives such as N-alkylamino substituted derivatives (where the alkyl groups preferably have 1 or 2 carbon atoms); and N,N-dialkyl substituted derivatives where the two alkyl groups may be but need not necessarily be identical to each other

(iii) phthalimide or substituted derivatives thereof

For the residues identified under (i) and (ii), the saturated aliphatic linker will preferably be bonded directly to the carbon atom of the aromatic ring. In the case of (iii) the linker will be bonded to the imide ring which (because the phthalimide

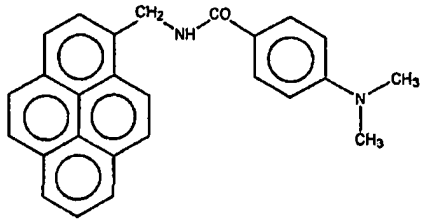
residue is the exciplex partner) does not constitute part of the saturated aliphatic linker.

It is particularly preferred in accordance with the invention that the donor exciplex partner is a 1-pyrenyl group and the acceptor is an amino, N-alkylamino or N,N-dialkylamino substituted benzene or naphthalene nucleus. Preferably the amino (or alkyl substituted amino group) is of the 4-position relative to the position at which the linker is bonded to the benzene or naphthalene. The alkyl groups may be methyl or ethyl groups. For an N,N-dialkylamino compound the alkyl group may be the same or different.

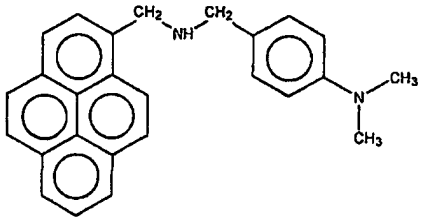
The invention will be illustrated by the following non-linking Examples which are studies based on the compounds SM1-8 for which the structures are shown in Scheme 1. The synthesis of compounds SM1-8 is described in the Appendix A. Compounds SM-2, SM-3 and SM-8 are in accordance with the invention. The other compounds are comparative.

Scheme 1

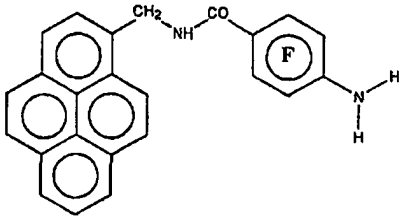
SM-1



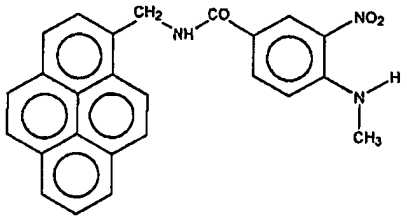
SM-2



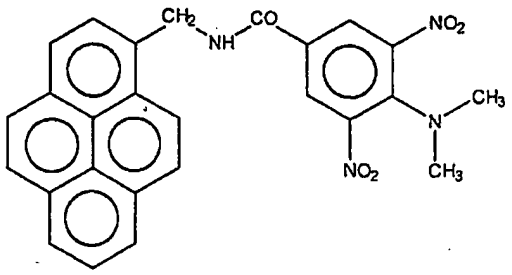
SM-5



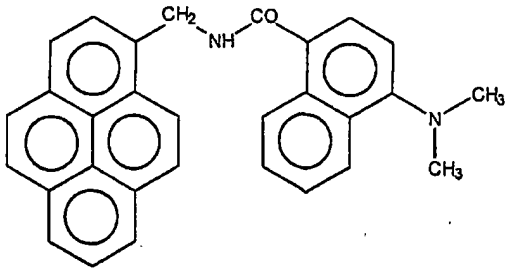
SM-7



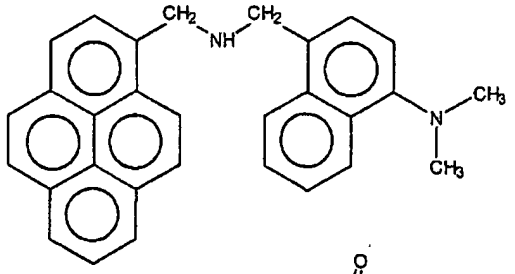
SM-6



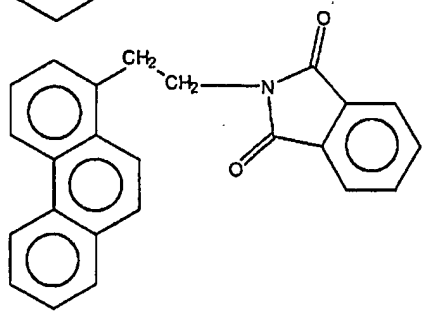
SM-4



SM-3



SM-8



1. Example 1 – Study of SM-2

1.1 Exciplex Formation by SM-2 in Non-Polar and medium Polarity Solvents.

The fluorescence characteristics of SM-2 (a compound in accordance with the invention) in a variety of non-polar and medium polarity solvents were studied (using the procedures described more fully below) and the results are set out in Table 1, and shown in Figures of the accompanying drawings.

Figure 1 represents the emission spectra of 1-methylamino-pyrene (solid curve) and **SM-2** (dashed curve (ii)) in toluene (the dielectric constant is 2.34 [26]) at 10^{-5} M concentration (excitation wavelength was 342 nm). The λ_{max} for monomer emission was 378 nm for both 1-methylamino-pyrene and for **SM-2**. An additional emission band with λ_{max} 489 nm was observed for **SM-2.** This was attributed to intramolecular exciplex formation between the **Pyr** (pyrene) and **DMA** (dimethyl aniline) moieties of **SM-2**. This new emission band could not be attributed to intermolecular excimer formation between the **Pyr** moieties of two molecules of **SM-2** because no excimer formation was discovered for 1-methylamino-pyrene in a control experiment performed at the same concentration and under identical conditions (Figure 1, solid curve). It should be emphasised that addition of dimethylaniline (10^{-5} M final concentration) to the toluene solution of 1-methylamino-pyrene (10^{-5} M) in a separate control experiment did not result in intermolecular exciplex formation. In fact, intermolecular exciplex formation started to be observable only after the addition of a 100-fold excess of dimethylaniline (10^{-3} M final concentration) to the toluene solution of 1-methylamino-pyrene (10^{-5} M) (data not shown).

SM-2 was also found to form an intramolecular exciplex in 100% THF (Figure 2, solid curve) with emission λ_{max} 522 nm and excitation wavelength of 342 nm (the dielectric constant for THF is 7.58 [27]). It was found that the λ_{max} of the

exciplex emission depends strongly on solvent polarity (Table 1). Exciplex emission was also detectable in aqueous-THF solution (H_2O: THF (2:8)). However, the presence of water decreased significantly the ability of **SM-2** to form the exciplex based on relative intensity (Figure 2, dashed curve). In fact, in 100% THF the I_{EX}: I_M ratio was 0.9:0.1 while in H_2O: THF (2:8) mixture the I_{EX}: I_M ratio was inverted to 0.65:0.35, where I_{EX} and I_M are the relative integral intensities of exciplex and monomer emission, respectively (Table 1).

Relatively weak intramolecular exciplex formation for **SM-2** was found to occur in medium polarity solvents, e.g. in DMF (the dielectric constant is 36.7 [27]). (Figure 3, , Table 1). However, the addition of H_2O to this DMF solution (to give H_2O:DMF (2:8), which has a net dielectric constant of 45.07 at 20°C) almost completely suppressed exciplex formation (Figure 3,). This is in accord with literature reports of the effects of increasing water percentage in mixed solvent systems [12;17]

No exciplex emission was observed for **SM-2** in acetonitrile (dielectric constan

t 35.9 [27]), or in acetonitrile /water solutions (Table 1).

Table 1. Fluorescence characteristics of monomer and exciplex emission obtained for **SM-2** in different solvents and solvent mixtures. I_{EX} and I_M values cannot be compared between solvent systems in this table.

Solvent	Refractive index, η	Dielectric constant,ε (debye)	λ_{max}(nm) monomer	λ_{max}(nm) exciplex	I_M [d]	I_{EX} [e]
Toluene	1.4893	2.34 [a]	378	489	0.31	0.69
THF	1.4040	7.58 [b]	378	522	0.10	0.90
THF/H_2O (80%/20%)	1.3892	21.77 [c]	374	522	0.35	0.65
DMF	1.4270	36.7 [b]	378	535	0.86	0.14
DMF/H_2O (80%/20%)	1.4083	47.05 [c]	378	535	0.99	0.01
Acetonitrile	1.34576	35.9 [b]	378	-	1.00	-

a) Dielectric constant was taken from [26];

b) Dielectric constant was taken from[27];

c) Dielectric constant was calculated from the equation: $\varepsilon^* = A\varepsilon_M + B\varepsilon_N$, where ε_1 and ε_2 – are the respective dielectric constant of the solvents M and N, and where A and B are is a proportions of the solvent M and N, respectively. For refractive indices of mixed media a similar equation was used.

d) Normalised fractional intensity of the monomer emission, relative to exciplex in the same experiment (sum of intensity for monomer plus exciplex = 1.0)

e) Normalised integral intensity of the exciplex emission, relative to monomer emission.

1.2 Exciplex Formation by SM-2 in Aqueous Solutions

The fluorescence characteristic of SM-2 in aqueous solutions (the dielectric constant for water is 78.54[26]) was tested and the results are set out in Table 2 and shown in Figures of the accompanying drawings.

Figure 7 represents the emission spectra of 1-methylamino-pyrene (dashed curve) and **SM-2** (solid curve) in 0.01 M Tris buffer (pH 9.0) prepared in H_2O, at 10^{-5} M concentration (excitation wavelength was 350 nm). The λ_{max} for monomer emission was 378 nm for both 1-methylamino-pyrene and **SM-2**. An additional intense emission band with λ_{max} of 484 nm was observed for **SM-2,** which is attributed to intramolecular exciplex formation between **Pyr** and **DMA** moieties of **SM-2**. This new emission band is unlikely to arise from **intermolecular** excimer formation between the **Pyr** moieties of **SM-2** because no excimer formation was discovered for 1-methylamino-pyrene in a control experiment performed at the same concentration and under identical conditions (Figure 7). This is the first reported case of intramolecular exciplex formation in aqueous solution that has not required an additive, such as cyclodextrins [20] or polyanions, such as chondroitins [23].

At a pH 6, no emission from **SM-2** was observed (see Table 2).

Table 2. Fluorescence characteristics of monomer and exciplex emission obtained for **SM-2** in water.

Solvent	Refractive index, η	Dielectric constant,ε (debye)	λ_{max}(nm) monomer	λ_{max}(nm) exciplex	I_M [d]	I_{EX} [e]
10mM,Tris buffer (pH 9.0)	1.3330 (water)	78.54 [a]	378	484	0.05	0.95
10mM,Tris buffer (pH 6)	1.3330 (water)	78.54 [a]	378	-	1.00	0

a) Dielectric constant was taken from [26];

d) Normalised fractional intensity of the monomer emission, relative to exciplex in the same experiment (sum of intensity for monomer plus exciplex = 1.0)

e) Normalised integral intensity of the exciplex emission, relative to monomer emission.

We also carried out a preliminary investigation of the influence of salt concentration on exciplex formation in aqueous solvents, close to physiological conditions. Figure 8 shows emission spectra of **SM-2** in 0.01M Tris buffer (pH 9.0) in the absence (solid curve) and in the presence (dashed curve) of 0.1M NaCl. (A 5% decrease of the fluorescence intensity after the addition of 100 µl of 2M NaCl into the 2 ml cuvette can be attributed to the decrease in concentration of **SM-2**). Figure 8 shows that the presence of NaCl slightly quenched exciplex emission. The decrease in the intensity of exciplex emission was found to be 10% (after concentration correction). However, under approximately physiological conditions (0.1M NaCl, 0.01M sodium phosphate buffer, pH 9.0) intramolecular exciplex formation is still easily detectable for system SM-2.

1.3 Effect of pH on Exciplex Formation by SM-2 in Aqueous Solutions

The effect of pH on the fluorescence characteristics of SM-2 in aqueous solution (dielectic constant for water in 78.54[26]) was tested and the results are set out in Table 3 and shown in Figures of the accompanying drawings.

The pH-dependence of exciplex formation in aqueous solutions was analysed in a preliminary scouting study for **SM-2** (10^{-5} M) in Tris buffer (0.01 M) with a pH range of 5.5 – 12.7. The data on pH-dependence of the normalised intensity of monomer and exciplex emission are presented in Table 3.

Table 3. pH-dependence of normalised fluorescence intensities of monomer and exciplex emissions for **SM-2** (10^{-5} M) in 10 mM Tris buffer.

pH	I_M [a)]	I_{EX} [b)]
	378 nm	484 nm
11.3	0.50	0.50
10.12	0.56	0.44
9.55	0.54	0.46
9.18	0.55	0.45
8.96	0.60	0.40
8.44	0.59	0.41
8.12	0.61	0.39
7.72	0.71	0.29

7.45	0.75	0.25
7.20	0.87	0.12
6.99	0.98	0.02
6.34	1.00	0
6.00	1.00	0

a) – Normalised intensity of monomer emission at 378 nm; normalised such that the sum of monomer and exciplex emission intensities for any given pH equal 1.00

b) - Normalised intensity of exciplex emission at 484 nm;

The series of emission spectra of **SM-2** versus pH is shown in Figure 21 ($\lambda_{excitation}$, 350 or 340nm). In acidic and neutral media (pH ≤ 7.0) **SM-2** exciplex emission is not detected in aqueous solution and this is consistent with the known prior art on exciplexes in polar media. Surprisingly, increase of pH above 7.0 results in exciplex emission. Moreover, the normalised intensity of exciplex emission I_{EX} increases with the increase in pH, reaching a plateau at around pH 9.0. A plot of relative exciplex emission intensity versus pH (Figure 21a) is overlaid in this Figure by a theoretical ionisation curve corresponding to a single acid ionisation of pK_a 7.58 ± 0.10.

1.4 Effect of Some Other Component of the Medium

Figure 22 gives a pictorial representation of the importance of alkaline pH for exciplex emission. In pure water (pH 6.0, curve (xi)) and in 0.01M sodium phosphate buffer (pH 7.0, curve (xii)) **SM-2** presents only monomer emission, λ_{max} 378 nm. The addition of a drop of concentrated NaOH into each of the above solutions (final pH ~ 14) resulted in the appearance of intense exciplex emission (curves (xiii) and (xiv) corresponding to water and to buffer systems, respectively). The curve (xv) corresponding to the emission spectrum of **SM-2** in Tris buffer (pH 10.0) is shown for

comparison. These experiments clearly show that exciplex emission is strongly affected by the pH of the solution, but that it is not very sensitive to buffer composition. Based on the above data, it is reasonable to propose that exciplex formation in aqueous solvents is strongly influenced by the extent of $-N(CH_3)_2$ protonation, namely, by the pK_a of the $-N(CH_3)_2$ group, or possibly by the protonation properties of the amino group in the linker itself. Possibly, the protonation of nitrogen atom of the $-N(CH_3)_2$ group and/or –NH-linker site completely suppresses the charge-transfer between **DMA** (donor) and **Pyr** (acceptor).

1.5 Exciplex formation in aqueous solutions in the presence of β-cyclodextrin

β-Cyclodextrin is known to form hydrophobic cavities in which intramolecular exciplexes can be formed by some small molecules in polar solvents (even in aqueous solutions) [20;21]. Thus, we studied the possibility of exciplex formation by **SM-2** in aqueous solutions in the presence of β-cyclodextrin. This test experiment was performed in 0.01 M Tris buffer (pH 10.0), using conditions (concentration of **SM-2**=10^{-4} M) which were shown earlier to be suitable for exciplex formation (Figure11, curve (i). Surprisingly, the addition of β-cyclodextrin (final cuvette concentration 0.01M) completely suppressed exciplex formation (Figure 11, curves (ii) and (iii)). This can be explained by the large dimensions of the **Pyr** acceptor component of **SM-2** preventing the insertion of **Pyr** into the β-cyclodextrin cavity; the small **DMA** moiety may be located inside the β-cyclodextrin.

2. Example 2- Study of SM-3

2.1 Exciplex Formation by SM-3 in Non-Polar and Medium Polarity Solvents

SM-3, which contains the pyrene group as an acceptor and DMN (N,N-dimethyl naphthalyl) group as a donor, was found to form an intramolecular exciplex in toluene or acetonitrile media.

Figures 4 and 5 represent the emission spectra of 10^{-5} M **SM-3** and **SM-4** solid and dashed curves, respectively) (blue) (curve (ix)) in toluene and in THF respectively (excitation wavelength was 344 nm). The value of λ_{max} for monomer emission of the **SM-3** was 383 nm in both toluene and THF, while λ_{max} for exciplex emission was 515 nm in toluene and 530 nm in THF (Table 4). As seen previously for **SM-2**, increase of solvent polarity results in a red shift of the exciplex λ_{max}. Additionally, the λ_{max} for exciplex emission for **SM-3** was red-shifted, compared with the **SM-2** under similar conditions. The proportion of fluorescence emission arising from exciplex relative to monomer for **SM-3** is slightly less than for **SM-2,** both in toluene and THF.

Table 4. Fluorescence characteristics of monomer and exciplex emission obtained for **SM-3** in different solvents. I_{EX} and I_M values cannot be compared between solvent systems in this table.

Solvent	Dielectric constant (debye)	λ_{max}(nm) monomer	λ_{max}(nm) exciplex	I_M [c]	I_{EX} [d]
Toluene	2.34 [a]	383	515	0.48	0.58
THF	7.58 [b]	383	530	0.57	0.43
Tris pH 10	78.54 [a]	377	482	0.05	0.95
pH 6.34tris	78.54 [a]	377	482	0.11	0.89

a) Dielectric constant was taken from [26];

b) Dielectric constant was taken from[27];

c) Normalised integral intensity of the monomer emission, relative to exciplex

d) Normalised integral intensity of the exciplex emission, relative to monomer

2.2 Exciplex Formation by SM-3 in Aqueous Solutions

The fluorescence characteristics of SM-3 in aqueous solutions (the dielectric constant for water is 78.54[26]) was tested and the results are set out in Table 5 and shown in Figures of the accompanying drawings.

Figure 9 shows emission spectra of **SM-3** (10^{-5} M) in 0.01 M Tris buffer, pH 9.0, pH 7.0 and pH 6.5, as indicated (excitation wavelength was 342 nm). The λ_{max} for monomer emission was 377 nm for **SM-3**. An additional intense emission band with λ_{max} of 482 nm was observed for **SM-3**, which is attributed to **intramolecular** exciplex formation between **Pyr** and **DMA** moieties of **SM-3**. It is seen from Figure 9 and Table 5 that at pH 6.5 the normalised integral intensity of the exciplex emission

for **SM-3** was 0.89.

Table 5. Fluorescence characteristics of monomer and exciplex emission obtained for **SM-3** in water.

Solvent	Dielectric constant (debye)	λ_{max}(nm) monomer	λ_{max}(nm) exciplex	I_M [c)]	I_{EX} [d)]
Tris pH 9.0	78.54 [a)]	377	482	0.05	0.95
pH 6.5(tris)	78.54 [a)]	377	482	0.11	0.89

a) Dielectric constant was taken from [26];

c) Normalised integral intensity of the monomer emission, relative to exciplex

d) Normalised integral intensity of the exciplex emission, relative to monomer

3. Example 3 – Study of SM-8

3.1 Exciplex Formation by SM-8

SM-8 also gives an intramolecular exciplex which emits in aqueous media (see Figure 10).

4. Example 4 – Influence of Linker Structure on Ability to form Exciplexes

The influence of linker flexibility on exciplex formation was first studied for **SM-1** and **SM-2** in toluene (Figure 6) at concentrations of 10^{-5} M. Comparison of the emission spectra of **SM-1** (Figure 6) (dashed curve) and **SM-2** (Figure 6) (solid curve) showed that the replacement of the flexible **–CH_2-NH-CH_2-** linker by the more rigid **–CH_2-NH-CO-** linker (with planar *trans* amide group) resulted in complete loss of

exciplex emission for **Pyr–CH_2-NH-CO-DMA**. These experimental observations are in accord with the data obtained by molecular modelling for **SM-1** and **SM-2** (see Example 5). Similar results were obtained for **SM-4** in toluene and THF (Figure 4 and 5, respectively). **SM3** and **SM4** are analogues, but **SM-3** possesses a flexible **-CH_2-NH-CH_2-** linker instead of a rigid **–CH_2-NH-CO-** linker as in **SM4**.

It should be emphasised that both **SM-1** and **SM-4** displayed different and completely surprising behaviour in aqueous conditions compared to non-polar solvents.

Other SM systems (**SM-5, SM-6 and SM-7**) with **–CH_2-NH-CO-** linker groups were tested for their abilities to form exciplexes in toluene. None of these compounds showed exciplex formation, even in non-polar conditions. The reason for this is the presence of the non-flexible **–CH_2-NH-CO-** linker group connecting the **Pyr** with **DMA** or **DMN** moieties. An additional reason for the **SM-5** and **SM-6** compounds may be the substitution of -N–$(CH_3)_2$ groups by the -NH_2 and -NH–(CH_3) groups in former and latter, respectively. It was shown in a separate experiment that the ability of aniline derivatives to form an intermolecular exciplex with pyrene decreased in the order: **DMA**, aniline, **DEA**.

5. Example 5 – Molecular Modelling and Compound Design

Compound design was performed with the assistance of molecular modelling using SYBYL 6.4.2 software (TRIPOS force field) on a Silicon Graphics Indy R4400 workstation. The structural parameters and charge distribution for the components of the system were calculated using the MOPAC module (SYBYL 6.4.2). All molecular modelling was effected without explicit inclusion of solvent.

It was appeared by computer modelling that linker length and chemical structure could be crucial for exciplex formation. Thus, a short linker group –CH_2-NH-CO- containing a planar, non-flexible amide bond prevents the formation of any stacking orientation between the two partners for the **Pyr**–CH_2-NH-CO-**DMA** system

(SM-1).

Figure 18 (left) shows the starting structure of the **SM-1** molecule with a restrained parallel orientation of the **Pyr** and **DMA** partners which are 3.04 Å apart. It should be noted that this artificial, restrained structure possesses a very high total energy (E = 25.802 kcal/mol) due to high values of Angle Bending energy, and especially of Torsional Energy terms. Minimisation of this starting structure (initial Simplex optimisation, followed by the Powell method with a gradient of $0.001 kcalmol^{-1}$, TRIPOS force field) resulted in a low-energy final structure (Figure 18 (right)) with non-stacking orientation of **Pyr** and **DMA** partners that are 7.7 Å apart (E = 7.29 kcals/mol). This result is consistent with the view that parallel orientation of the donor and acceptor partners favours exciplex emission. This is geometrically forbidden for **Pyr**–CH_2-NH-CO-**DMA**, in turn suggesting that exciplex formation is likely to be less favoured in this case.

The substitution of the –CO group by the more flexible -CH_2 group increases the ability of **Pyr**–CH_2-NH-CH_2-**DMA** (**SM-2**) to form a stacking structure with parallel orientation of the closely located **Pyr** and **DMA** components, perhaps due to a higher degree of conformational freedom of linker group.

Figure 19 shows the two low-energy structures of **SM-2** representing the stacking and non-stacking orientation of the **Pyr** and **DMA** partners, respectively. It is seen that a stacking structure with close location of exciplex partners (3.67 Å) was characterised with a lower total energy than the non-stacking structure (the distance between **Pyr** and **DMA** was 7.85 Å). This result clearly suggests the potential ability of **Pyr**–CH_2-NH-CH_2-**DMA** to form an exciplex structure.

Figure 20 summarises the results of molecular modelling obtained for **SM-2** (left) and **SM-1** (right), showing the most favourable low-energy conformations for these molecules.

Thus, the results of molecular modelling are consistent with the supposition that the linker groups need to be flexible, and possess a high degree of rotational

freedom to allow parallel orientation of the partner chromophores. Rigid, non-flexible fragments in linker groups impose strict conformational rules that in general disfavour formation of a putative parallel exciplex structure.

6. Example 6 – Absorption and Excitation Spectra of SM-1, SM-2 and SM-3

To characterise the excited and ground states of some SMs we recorded the UV-visible absorption spectra as well as excitation spectra for both monomer and exciplex band emission (Figures 12 - 17).

Figure 12 shows absorption spectra of **SM-2, SM-1**, 1-methylamino-pyrene and naphthalene recorded in toluene at 10^{-5} M concentration. It is seen that low-wavelength bands of the pyrene moiety (310-350 nm) of both **SM-1** and **SM-2** show close spectral similarity to the respective absorption band of 1-methylamino-pyrene, apart from the slight red-shift of the λ_{max} of both **SM-1** and **SM-2** (346 nm) as compared with λ_{max} of free 1-methylamino-pyrene (344 nm), which may be attributed to the difference in chemical structures of these compounds. This result indicates the similarity of ground states for the pyrene moieties for **SM-1, SM-2** and 1-methylamino-pyrene. These data are in accordance with the excitation spectrum of **SM-2** recorded in toluene (Figure 13). The excitation spectrum for the exciplex fluorescence emission band at 504 nm is identical to the excitation spectrum for monomer fluorescence emission band at 393 nm, showing that the exciplex responsible for the lower-wavelength fluorescence band is dissociated in the ground state.

A slightly different situation and thus surprising was found for **SM-2** in aqueous solutions. The UV-visible spectrum of SM-2 (Figure 14) in 10 mM Tris buffer (pH 9.0) showed significant spectral broadening and a 13 nm red-shift of the lower-wavelength band (λ_{max}, 354 nm) compared with 1-methylamino-pyrene (λ_{max}, 341 nm, black curve). However, no additional absorption band was observed for **SM-2** under the above conditions. It should be noticed that no spectral broadening and no

significant red-shift were observed for the absorption spectrum of **SM-2** recorded at pH 6.34 (magenta) when no exciplex was formed. These data suggest some charge-transfer interactions between donor and acceptor groups in their ground states. Compare reports for intramolecular exciplexes formed from anthracene [28].

These data are correlated with the excitation spectra recorded for **SM-2** in 10 mM Tris buffer (pH 9.0) (Figure 15). The excitation spectrum for the exciplex fluorescence emission band at 490 nm is significantly broadened and red-shifted compared to the excitation spectrum for the monomer fluorescence emission band at 375 nm, showing the charge-transfer nature of the exciplex in aqueous solutions.

Similar results were obtained for **SM-3** both in toluene and aqueous solution (Figures 16 and 17, respectively).

7. Example 7 – Labelling of Primers

(i) The 8-mer oligodeoxyribonucleotide pTGTTTGGC was condensed through its 5'-phosphate site with N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (**3**) to produce the labelled oligonucleotide designated herein as **3-pTGTTTGGC.** The synthetic procedures used were as described in Appendix A.

The exciplex emission spectrum of **3** in pH 9 tris buffer is illustrated in Fig 23. For **3-pTGTTTGGC** a long tail of emission was seen in the region of 480 nm, with only a weak peak in this region at pH 7 or 10 (**Figure 24**). The effects of added organic solvent on the exciplex emission of **3-pTGTTTGGC** (**Figure 25**) were tested: the addition of (i) acetonitrile or (ii) methanol (to 50% v/v) to **3-pTGTTTGGC** in pH 8.5 Tris buffer gave no significant increase in emission at 480 nm, although there was some change detected for (iii) tetrahydrofuran. The spectrum of **3-pTGTTTGGC** in (iv) pure tetrahydrofuran (**Figure 25**) shows strong emission at 485 nm, typical of the exciplex.

(ii) The 8-mer dCGATTCTGp was labelled on the 3' phosphate with compound **3** to produce the labelled oligonucleotide designated herein as **dCGATTCTGp-3**. The emission spectra of **dCGATTCTGp-3** are shown in **Figure 26**: the exciplex emission band around 485 nm is clearly seen in (v) pure tetrahydrofuran and in (vi) a medium prepared from 0.01M Tris buffer at pH 8.8 (10% v/v) and tetrahydrofuran (90% v/v). The exciplex emission spectrum in 0.01m Tris buffer at pH 8.8 is shown in Fig 4 as curve (vii). With reference to **Fig 27**, the exciplex emission band at 480 nm is seen to increase in distinctness on going from (viii) 50, to (ix) 75 to (x) 92.5 % THF in the Tris buffer pH8.8 (in these experiments the solution was diluted by addition of THF and so the concentration of fluorophore also decreased).

Summary

1. A number of examples are given whereby intramolecular exciplexes can be constructed such that their exciplex properties are not quenched in aqueous or other highly polar media.

2. This allows the use of aqueous media to make use of any property of the exciplex that previously was restricted to non or low percentage aqueous environments, such as lasers, dyes to act as fluorescent or circular dichroism detectors when the intramolecular exciplex analogues have been used to label other materials including, proteins, nucleic acids and (oligo)nucleotides and their analogues, such as PNAs, glycoproteins, solid materials including polymers, ceramics, transistors, semiconductors, insulators, conductors, glasses.

3. It appears from the observed data that the relative fluorescence emission in the region of the inflexion in the curve is MORE sensitive to pH than predicted by the Henderson-Hasselbach equation. This is even more surprising and novel and means that the system may provide the basis of an even more pH-sensitive pH device or pH switch. The solid curve shown in the Figure indicates what would be the behaviour on pH change if Henderson-Hasselbach behaviour were followed.

4. In contrast to **SM-2**, the ability of **SM-3** to give exciplex emission is less sensitive to the pH of the solution. It is seen from Figure 7 and Table 1 that even at pH 6.5 the normalised integral intensity of the exciplex emission for **SM-2** was 0.89.

5. Not all exciplexes which we have made and shown to emit in aqueous media show pH effects and thus the observations above are surprising (compare **SM-8**, Figure 10).

4. APPENDIX A. SYNTHETIC METHODS

A.1 Synthesis of SM-1 and SM-2

(i) 4-Dimethylamino-N-(1-pyrenemethyl)benzamide, SM-1

A solution of 1-pyrenemethylamine hydrochloride (0.5g, 1.865mmol) in dichloromethane (150ml) was treated with triethylamine (0.45g, 4.47mmol) and stirred for 30 min at room temperature. 4-Dimethylaminobenzoyl chloride (0.34g, 1.865mmol) in dichloromethane (50ml) was added dropwise over 30 min. and the mixture stirred for 5 hrs until TLC (DCM/EtOAc, 9:1) indicated completion. The reaction mixture was washed successively with 1N HCl (1x100ml), 1N $NaHCO_3$ (3x100ml), H_2O (100ml) and brine (100ml). The organic layer was dried over anhydrous Na_2SO_4. Evaporation of solvent afforded crude product which was recrystallised from ether to give a white solid (0.358g, 50.59%); m.p: 204-206°C. ^{1}H-NMR (δ_H, $CDCl_3$): 2.97 (s, 6H, -NC<u>H</u>$_3$); 5.34 (d, 2H, PyC<u>H</u>$_2$N-); 6.34 (bs, 1H, -N<u>H</u>CO-); 6.69 (d, 2H, -Ar); 7.71 (d, 2H, -Ar); 7.97-8.36 (m, 9H, -Py), ^{13}C-NMR (δ_C, $CDCl_3$): 40.31; 42.63; 111.83; 123.44; 124.69; 124.88; 125.31; 126.25; 127.31; 127.56; 128.56; 129.25; 129.50; 130.81; 131.88; 167.19.

(ii) N-(4-Dimethylaminobenzyl)-N-(1-pyrenemethyl)amine, SM-2

To a solution of 4-dimethylamino-N-(1-pyrenemethyl)benzamide (0.1gr, 0.26mmol) in dry ether (15ml) was added $LiAH_4$ (0.075g, 2mmol). The mixture was refluxed until TLC monitoring showed consumption of all of the starting material. The mixture was then stirred at room temperature overnight. After washing with water (2x20ml), the ethereal solution was dried over anhydrous $MgSO_4$. The resulting oily residue after evaporation was purified by flash chromatography on a column of silica gel with ether to afford a light yellow product as an oil (0.07g, 70%). The oil was dissolved in DCM/ether and HCl(g) was bubbled through to form the corresponding dihydrochloride salt as a white solid; R_f: 0.21 (Et_2O). ^{1}H-NMR (δ, $CDCl_3$) 2.09 (bs, 1H, -N<u>H</u>); 2.89 (s, 6H, 2-NC<u>H</u>$_3$); 3.83 (s, 2H, -NC<u>H</u>$_2$Py); 4.41 (s, 2H, -NC<u>H</u>$_2$Ar); 6.67-6.74 (dd, 2H, -Ar); 7.21-7.27 (dd, 2H, -Ar); 7.80-8.47; (m, 9H, -

Py).

A.2 Synthesis of SM-5

4-Amino-2,3,5,6-tetrafluoro-N-(1-pyrenemethyl)benzamide, SM-5

A solution of 1-pyrenemethylamine hydrochloride (0.2g, 0.72mmol) in DCM (15ml) and triethylamine (0.11ml, 0.86 mmol) was stirred for 30 min at room temperature. To this mixture was added 4-amino-2,3,5,6-tetrafluorobenzoic acid (0.157g, 0.76mmol,) in DCM (15ml) and 4-dimethylaminopyridine (0.09g, 0.74mmol). Finally N,N-diisopropylcarbodiimide (0.12ml, 0.77mmol) was added drop-wise at 0°C. The mixture was refluxed for 3hrs and stirring continued overnight at room temperature. After washing with $NaHCO_3$ (10%, 30ml) and water (2x30ml), the organic layer was dried over $MgSO_4$. Evaporation of solvent and purification of residue afforded the pure product as a cream solid (0.19g, 60%), R_f : 0.55 (DCM/EtOAc, 17:1). ^{1}H-NMR (δ, $CDCl_3$): 4.19 (bs, 2H, -N$\underline{H_2}$); 5.35-5.36 (d, 2H, PyC$\underline{H_2}$N); 6.28 (bs, 1H, -N$\underline{H}$CO-); 8.01-8.33 (m, 9H, -Py).

A.3 Synthesis of SM-7

4-Amino-3-nitro-N-(1-pyrenemethyl)benzamide, SM-7

A solution of 1-pyrenemethylamine hydrochloride (0.1g, 0.36mmol) in dichloromethame (10ml) and triethylamine (0.05ml, 0.36mmol) was stirred for 30 min at room temperature. To this mixture was added 4-amino-3-nitrobenzoic acid (0.07g, 0.38mmol) in dichloromethane (10ml) and 4-dimethylaminopyridine (0.044g, 0.36mmol). Finally N,N-diisopropylcarbodiimide (0.06ml, 0.38mmol) was added dropwise at 0°C. The mixture was refluxed for 3hrs and stirring was continued at room temperature overnight. After washing with $NaHCO_3$ (10%, 20ml) and water (2x20ml), the organic layer was dried on $MgSO_4$. Evaporation of solvent and purification of the residue with dichloromethane/EtoAc (3:1) on a column of silica gel afforded the pure product as a yellow solid (0.09g, 61%); R_f 0.44 (DCM/ EtOAc, 3:1). ^{1}H-NMR (δ, Acetone-d_6) : 4.95-5.02 (m, 2H, -N$\underline{H_2}$); 5.21-5.22 (d, 2H, -C$\underline{H_2}$N); 6.94-

6.98 (d, 1H, -Ar); 7.22 (bs, 1H, -NHCO); 7.86-8.42 (m, 10H, -Py, -Ar); 8.56-8.59 (d, 1H, -Ar).

A.4 Synthesis of SM-3 and SM-4.

(i) 4-N-Dimethylamino-(1-pyrenemethyl)naphthamide, SM-4

A solution of 1-pyrenemethylamine hydrochloride (0.2g, 0.72 mmol) in DCM (20ml) was treated with triethylamine (0.11ml, 0.79 mmol) and stirred for 30 min at room temperature. 4- Dimethylamino-naphthoic acid (0.194g, 0.76mmol, in DCM (20ml) and 4-dimethylaminopyridine (0.088g, 0.72 mmol) were added. To this solution, N,N-diisopropylcarbodiimide (0.12ml, 0.77 mmol) was added drop-wise at 0°C under nitrogen and the reaction mixture refluxed for 3hrs. The mixture was stirred overnight at room temperature under drying tube and then washed with 1N $NaHCO_3$ (50ml) and H_2O (2x50ml). The organic layer was dried over anhydrous $MgSO_4$. Evaporation of solvent gave the crude product and column chromatography on silica gel with DCM/EtOAc (10:1) afforded the pure product.

(ii) N'-4-Dimethylaminonaphthyl-N-(1-pyrenemethyl)amine, SM-3

In a 50ml flask, $LiAH_4$ (0.075g) was added to a solution of 4-dimethylamino-N-(1-pyrenemethyl)naphthamide (SM-3, 0.17g, 0.4mmol) in dry ether (30ml) and dry THF (15ml). The mixture was refluxed for 7 hrs. TLC monitoring showed all of the starting material was consumed. The reaction mixture was left stirred overnight at room temperature. Aqueous ammonium chloride was added and the extracted ethereal layer dried on $MgSO_4$. Column chromatoghraphy of the residue from evaporation on silica gel (DCM eluent) gave the pure product as a light brown liquid (0.065g, 40.6%), R_f 0.14 (DCM/EtOAc, 20:1). ^{1}H-NMR (δ_H ,$CDCl_3$): 1.32 (bs, 2H, -NH_2); 3.11 (t, 2H, ArCH_2); 3.20 (t, 2H, -CH_2NH); 7.49-7.66 (m, 5H, -Ar); 7.80 (d, 1H, -Ar); 8.06 (d, 1H, -Ar); 8.72-8.59 (dd, 2H, -Ar).

A.5 Synthesis of SM-6

4-Dimethylamino-3, 5-dinitro-N-(1-pyrenemethyl)benzamide, SM-6

A solution of 1-pyrenemethylamine hydrochloride (0.2g, 0.72 mmol) in DCM (20ml) was treated with triethylamine (0.11ml, 0.79 mmol) and stirred for 30 min at room temperature. 4- Dimethylamino-3,5-dinitrobenzoic acid (0.194g, 0.76mmol) in DCM (20ml) and 4-dimethylaminopyridine (0.088g, 0.72 mmol) were added. To this solution, N,N-diisopropylcarbodiimide (0.12ml, 0.77 mmol) was added drop-wise at 0°C under nitrogen and the reaction mixture refluxed for 3hrs. The mixture was stirred overnight at room temperature under drying tube and then washed with 1N $NaHCO_3$ (50ml) and H_2O (2x50ml). The organic layer was dried over anhydrous $MgSO_4$. Evaporation of solvent and column chromatography of the crude product with hexane/DCM (1:4) afforded the pure product as a yellow solid (0.09g, 24.3%); m.p.: 221-224°C R_f: 0.23 (CDM). ^{1}H-NMR (δ_H, $CDCl_3$) : 2.82 (s, 6H, 2-$NC\underline{H}_3$); 5.32 (d, -$C\underline{H}_2NH$); 7.96-8.28 (m, 11H, -Ar, -Py).

Synthesis of 9-((N,N-dimethylamino)ethyl)phenanthrene (SM-9)

To prepare 9-((N,N-dimethylamino)ethyl)phenanthrene (SM-9), 2-(9-phenanthrenyl)ethanol was synthesised from 9-bromophenanthrene via the 9-phenanthrenyl magnesium bromide [29;30] and ethylene oxide[30;31]. This was converted to 2-(9-phenanthrenyl)ethyl chloride using thionyl chloride[31], Scheme X. Reaction of 2-(9-phenanthrenyl)ethyl chloride with potassium phthalimide in the presence of hexadecyltributylphosphonium bromide afforded the corresponding phthalimide, which was hydrolysed to 2-(9-phenanthrenyl)ethylamine using hydrazine hydrate[32], Scheme XI. Dimethylation of 2-(9-phenanthrenyl)ethylamine gave SM-9.

(i) 2-(9-Phenanthryl)ethanol

9-Bromophenanthrene (2g, 7.8mmol), magnesium turning (0.2g) and a catalytic amount of I_2 were suspended in dry THF (20ml). The mixture was refluxed

until all the magnesium had dissolved (1hr), the mixture cooled to -10° C and 1.4ml of ethylene oxide (0.48g, 10.8mmol) in dry ether (1.4ml from a solution contains 34gr ethylene oxide in 100ml dry ether) was added. After stirring at 0°C for 30 min and then at 40°C for 30 min, the solution was refluxed for 1hr, cooled to room temperature and then concentrated. Addition of 1N HCl (10ml) was followed by extraction with dichloromethane (2x20ml). The organic layer was washed with 1N NaOH (20ml) and H_2O and then dried over anhydrous $MgSO_4$. Evaporation of solvent and chromatography of crude product on silica gel with hexane/dichloromethane (3:7) afforded the pure product as a white solid (1.2g, 69.4%); m.p.: 86-87°C R_f : 0.34 (DCM). ^{1}H-NMR (δ, $CDCl_3$) : 1.53 (bs, 1H, O<u>H</u>); 3.42 (t, 2H, -C<u>H$_2$</u>-CH$_2$-O); 4.07 (t, 2H, -CH$_2$.C<u>H$_2$</u>-O); 7.58-7.72 (m, 5H, -Ar); 7.84-7.90 (d, 1H, -Ar); 8.11-8.16 (d, 1H, -Ar); 8.66-8.72 (d, 1H, -Ar); 8.74-8.80 (d, 1H, -Ar) .

(ii) 2-(9-Phenanthryl)ethyl Chloride

To a cold mixture of 2-(9-phenanthryl)ethanol (0.6g, 2.7mmol) and N,N-dimethylalanine (0.35ml) in dry toluene (2ml) cooled on ice water thionyl chloride (0.23ml, 3.15mmol) was added drop-wise. The red solution was refluxed until the evolution of sulfur dioxide ceased. On addition of water (20ml) an organic layer separated and was isolated with ether(30ml). After evaporation of ether and column chromatography on silica gel with hexane, the corresponding pure product was obtained as a white solid (0.5g, 77.0%); m.p.: 82-84°C ^{1}H-NMR (δ, $CDCl_3$) : 3.54-3.62 (t, 2H, -C<u>H$_2$</u>CH$_2$Cl); 3.86-3.93 (t, 2H, -CH$_2$C<u>H$_2$</u>Cl); 7.57-7.69 (m, 5H, -Phe); 7.83-7.85 (m, 1H, -Phe); 8.04-8.06 (m, 1H, -Phe); 8.65-8.67 (d, 1H, -Phe).

(iii) 2-(9-Phenanthrenyl)ethylphthalimide

A toluene solution (2ml) of 2-(9-phenanthrenyl)ethyl chloride (0.241g, 1mmol) and hexadecyltributylphosphonium bromide (0.051g, 0.1mmol), and potassium phthalimide (0.231g, 1.25mmol) were placed in a 5ml round-bottomed flask equipped with reflux condenser and magnetic stirrer, and heated at 100°C (bath temperature) under argon atmosphere with stirring. The extent of reaction was monitored by following the disappearance of starting material. After 20 hrs (95% conversion) water (30ml) was added to the cooled reaction mixture which was then extracted with ether (3 x 30ml). The combined ethereal layer was dried over $MgSO_4$ and evaporated under vacuum. The crude product was purified by column chromatography on silica gel with hexane/DCM (50:50) as eluent. The pure product was obtained as a white solid (0.24g, 56.6%), m.p: 140-143°C, R_f: 0.31 (hexane/DCM).

(iv) 4-N',N'-dimethylamino-N-(1-pyrenemethyl)naphthamide

Using the method described for 4-dimethylamino-3,5-dinitrobenzoic acid, the pure product (0.283g, 80%) was obtained as a white solid after column chromatography on silica gel with DCM/EtOAc (10:1) as eluent.

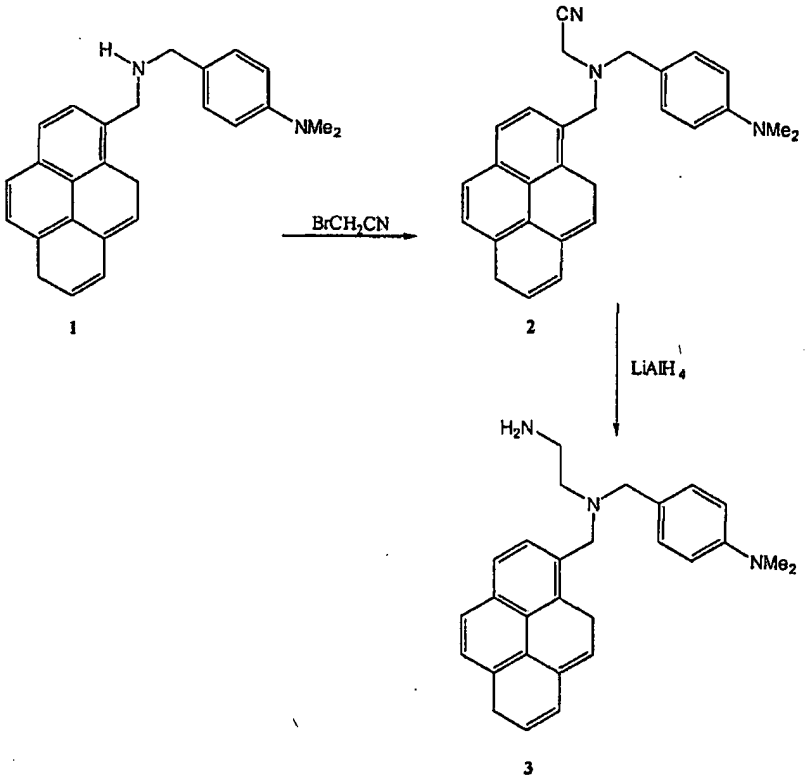
4-N',N'-dimethylaminonaphthyl-N-(1-pyrenemethyl)amine , SM-3

In a 50ml flask, $LiAH_4$ (0.075g) was added to a solution of 4-dimethylamino-N-(1-pyrenemethyl)naphthamide (0.17g, 0.4mmol) in dry ether (30ml) and dry THF (15ml). The mixture was refluxed for 7 hrs. TLC monitoring showed all of the starting material was consumed. The reaction mixture was left overnight in room temperature while it was stirred. An aqueous solution of ammonium chloride was added to the mixture and the ethereal layer extracted and dried over $MgSO_4$. Column chromatography of the residue on silica gel with DCM as eluent gave the pure product as a light brown liquid (0.065g, 40.6%), R_f:0.14 (DCM/EtOAc, 20:1), ^{1}H-NMR (δ_H

,$CDCl_3$): 1.32 (bs, 2H, -$N\underline{H}_2$); 3.11 (t, 2H, Ar$C\underline{H}_2$); 3.20 (t, 2H, -$C\underline{H}_2$NH); 7.49-7.66 (m, 5H, -Ar); 7.80 (d, 1H, -Ar); 8.06 (d, 1H, -Ar); 8.72-8.59 (dd, 2H, -Ar).

Synthesis of N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (3)

This was carried out according to the following Scheme



Synthesis of N-(cyanomethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine, **2**

Bromoacetonitrile (0.62mg, 0.52 mmol 0.035 ml) was added drop-wise to a stirred solution of N-(4-dimethylaminobenzyl)-N-1-pyrenyl)amine (0.19g, 0.52 mmol) and potassium carbonate (0.071g, 0.52 mmol) in acetone (0.5 ml) at 0°C. The reaction mixture was stirred at room temperature for 3h and then the solvent removed *in vacuo*. The resultant solid was triturated with water (15 ml) and extracted with diethyl ether (2 x 20 ml). The combined organic extracts were washed with water (2 x 10 ml), dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to give N-

(cyanomethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (**2**) as a yellow solid, which after chromatography on silica gel with dichloromethane and hexane as solvent (9:1) was isolated as a white product (0.08g, 38%).

Synthesis of N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine, **3**

To a solution of N-(cyanomethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (**2**, 0.06g, 0.15 mmol) in dry tetrahydrofuran (1 ml) was added $LiAlH_4$ (0.05g, 1.3mmol) and the mixture refluxed for 5 h after which the complex was decomposed by adding aqueous potassium hydroxide (2 ml, 40%). This mixture was extracted with diethyl ether (2 x 15 ml) after addition of a further aliquot of water (1 ml) and the combined ethereal extracts dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to give an oily yellow residue. Chromatography on Varian Mega BondElut SI(2g silica gel) with dichloromethane and a few drops of aq. NH_4OH yielded **3** as a yellow, oily product (0.05g, 81%).

DNA Labelling

To give **3-pTGTTTGGC**, the 8-mer oligodeoxyribonucleotide pTGTTTGGC was condensed through its 5'-phosphate site with N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-pyrenyl)amine (**3**) as follows. **3-pTGTTTGGC** was synthesised by a two-step procedure. First, the cetyltrimethylammonium salt of 5'pTGTTTGGC was obtained by stepwise addition of 8% aqueous cetyltrimethylammonium bromide (100µl, 20µlx5) to a solution of the lithium salt of the oligonucleotide (1µmol) in 0.3 ml of water, with centrifugation on each addition, until no more precipitation was observed. The supernatant was removed, the precipitate dried *in vacuo* overnight over P_2O_5, and the cetyltrimethylammonium salt of the oligonucleotide (1µmol) dissolved in 0.4 ml of DMF. Triphenylphosphine (13.2 mg, 50µmol) and 2',2'-dipyridyl disulfide (11.2 mg, 50 µmol) were added, and after 10 min 4-*N',N'*-dimethylaminopyridine (6.2 mg, 50µmol) was added. After 15 minutes incubation at 20°C, N-(2-aminoethyl)-N-(4-dimethylaminobenzyl)-N-(1-

pyrenyl)amine (**3**) (5.4mg, 20μmol) and triethylamine (28μl, 20μmol) was added and the reaction mixture incubated at 25°C for 1h, followed by precipitation by 25 ml of acetone containing 2% $LiClO_4$. The oligonucleotide conjugate (**3-pTGTTTGGC**) was separated from unreacted precursor (**3**) by reverse-phase HPLC (0 to 40% acetonitrile gradient) to give total yield of product of 90% based on starting oligonucleotide. The incorporation of the structure from 3 into the oligonucleotide was confirmed by its HPLC behaviour, and by UV-visible specrophotometry, giving a characteristic absorbance at 350 nm maximally for the chromophore corresponding to **3**.

*The synthesis of **DCGATTCTGp-3** was as described above but using dCGATTCTGp labelled on the 3' phosphate with compound 3.*

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Claims

1. A compound capable of forming an intramolecular exciplex on photoirradiation of the compound in water, said compound comprising two exciplex forming partners, one being a donor moiety and the other an acceptor moiety, each having at least one aromatic nucleus and being connected by a saturated aliphatic chain having the flexibility to allow said partners to come into exciplex forming relationship.

2. A compound as claimed in claim 1 wherein the saturated aliphatic chain comprises 2 to 6 saturated atoms as the link between the two exciplex forming partners.

3. A compound as claimed in claim 2 wherein the linker comprises 2 to 4 saturated atoms as the link.

4. A compound as claimed in claim 3 wherein the linker comprises 3 saturated atoms as the link.

5. A compound as claimed in any one of claims 1 to 4 wherein the saturated atoms of the linker are comprised wholly or partly of carbon atoms.

6. A compound as claimed in claim 5 wherein the carbon atoms are provided by methylene groups.

7. A compound as claimed in claim 2 wherein the linker is $-CH_2-CH_2-$ in which the free terminal buds are bonded to the exciplex forming partners.

8. A compound as claimed in claim 4 wherein the linker is $-CH_2-CH_2-CH_2-$ or $-CH_2-NH-CH_2-$ in which the free terminal bonds are bonded to the exciplex forming partners.

9. A compound as claimed in any one of claims 1 to 8 wherein the aromatic nucleic of the donor and acceptor moieties are selected from

(i) benzene, naphthalene, anthracene, phenanthrene, pyrene and chrysene, phenanthrene;

(ii) derivatives of residues of compounds identified under (i), particularly (but not exclusively) amino or substituted amino derivatives such as N-alkylamino substituted derivatives (where the alkyl groups preferably have 1 or 2 carbon atoms); and N,N-dialkyl substituted derivatives where the two alkyl groups may be but need not necessarily be identical to each other

(iii) phthalimide or substituted derivatives thereof

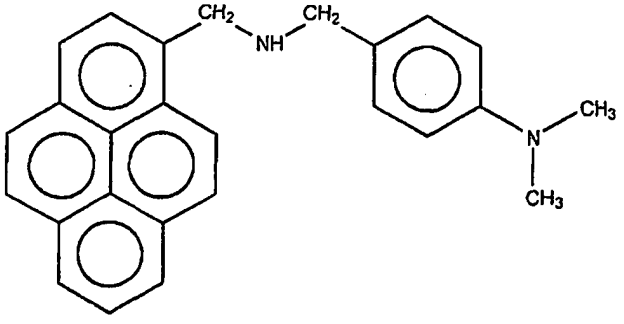
10. A compound as claimed in claim 9 wherein the donor moiety is a 1-pyrenyl group and the acceptor moiety is an amino, N-alkylamino, N,N-dialkylamino substituted benzene or naphthalene nucleus.

11. A compound as claimed in claim 10 wherein amino, N-alkylamino or N,N-dialkylamino group is at the 4-position at which the linker is bonded to the benzene or naphthalene nucleus.

12. A compound as claimed in claim 10 or 11 wherein the acceptor moiety is an N-alkylamino or N,N-dialkylamino substituted benzene or naphthalene nucleus and the alkyl groups have one to two carbon atoms.

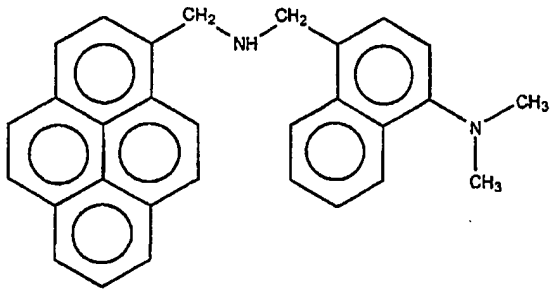
13. The compound

SM-2



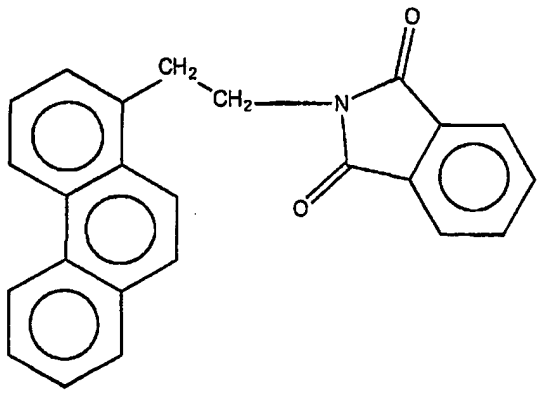
14. The compound

SM-3



15. The compound

SM-8



16. A compound as claimed in any one of claims 1 to 12 wherein the linker is substituted with a functional group permitting covalent attachment of the compound as a label to a molecule or other entity to be labelled.

17. A compound as claimed in claim 16 wherein the functional group is an amino group.

18. A molecule or other entity labelled with a compound as claimed in any one of claims 1 to 17.

19. An entity as claimed in claim 18 which is a labelled nucleic acid.

20. An entity as claimed in claim 18 which is labelled oligonucleotide.

21. An entity as claimed in claim 18 which is a labelled protein.

22. An entity as claimed in claim 18 which is a labelled ceramic, transistor, semiconductor, insulator or glass.

23. A method of generating an exciplex comprising irradiating a compound as claimed in any one of claims 1 to 17 or entity as claimed in any one of claims 19 to 22 with electromagnetic radiation of a wavelength appropriate for exciplex formation by said donor and acceptor moieties.

24. A method as claimed in claim 23 additionally comprising the step of detecting for exciplex formation.

25. A method of detection wherein there is used as a label a compound as claimed in any one of claims 1 to 17 and the method comprises irradiating a sample under test with electromagnetic radiation capable of providing exciplex formation in said label (if present) and detecting for exciplex formation.

26. A method as claimed in any one of claims 23 to 25 which is effected in a polar medium.

27. A method as claimed in claim 14 wherein the polar medium is an aqueous medium.

28. A method as claimed in claim 26 or 27 wherein the exciplex emission is pH sensitive.

29. A method as claimed in claim 28 which involves adjusting pH to obtain or modify exciplex emission.

30. A method as claimed in claim 28 which is effected to

(a) register pH changes in an environment such as a living or fixed cell, or in a curvette or other container including cell sorter devices, and be used in assays to other determinations, or

(b) induce a signal by manipulation of the pH of the environment externally, such as by added acid, base or other change in conditions which leads to a pH change.

Emission Spectra of SM-2 and Pyrenemethylamine in Toluene

(———) pyrenemethylamine;

(------) SM-2;

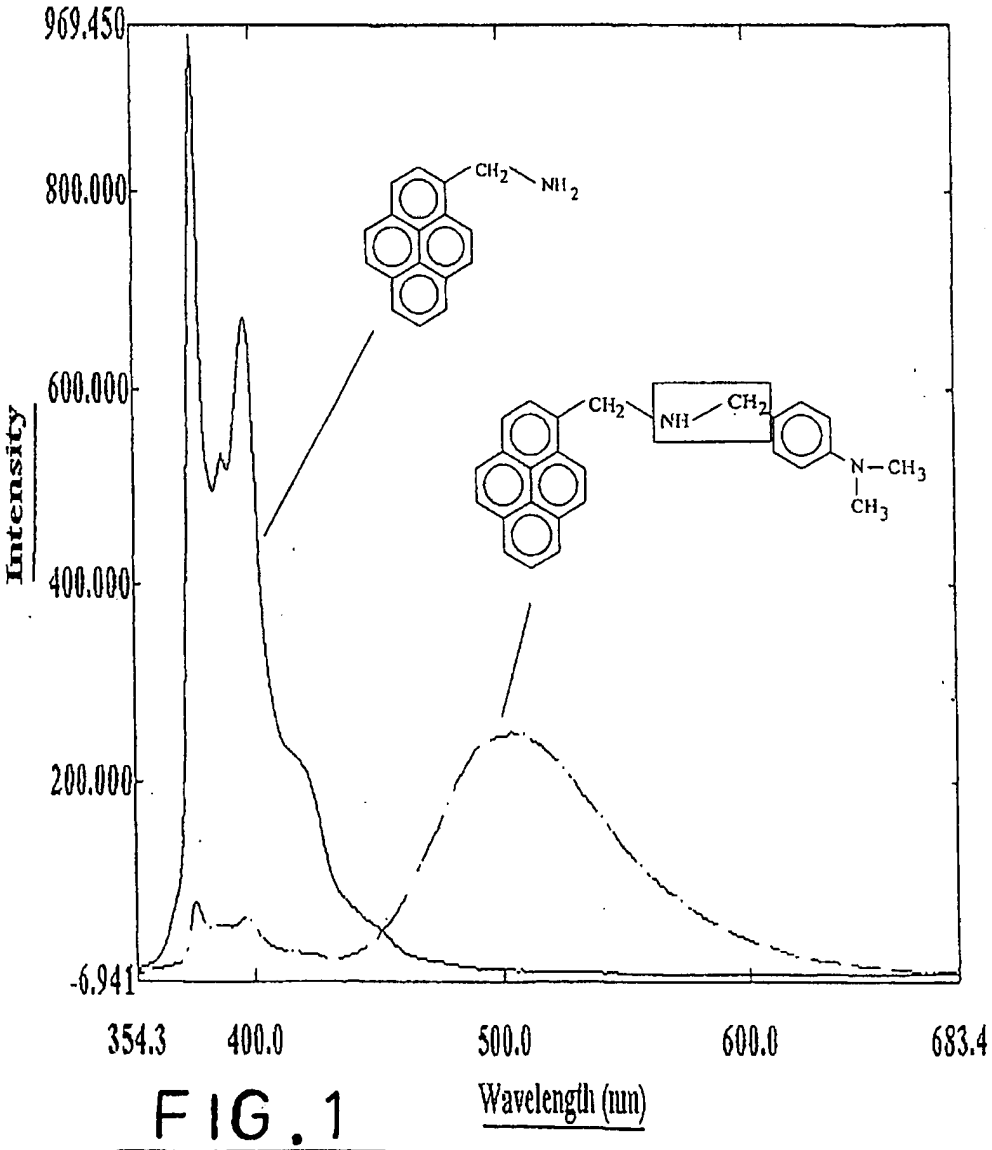


FIG. 1

Intramolecular exciplex emission obtained for SM-2 in THF (——) and in THF/water solution (80%/20%) (-----)

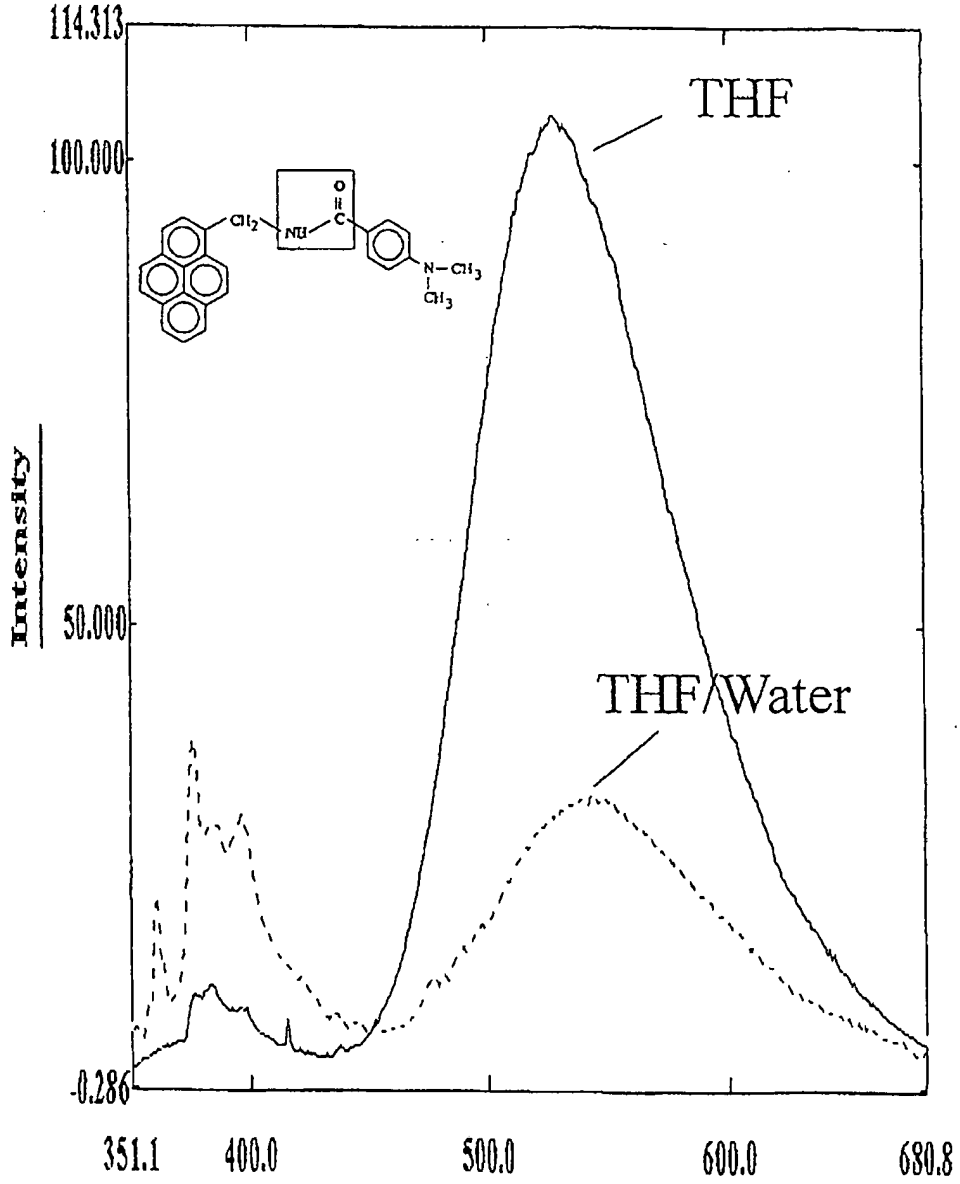


FIG. 2

SM-2 in DMF and in DMF/Water Solution

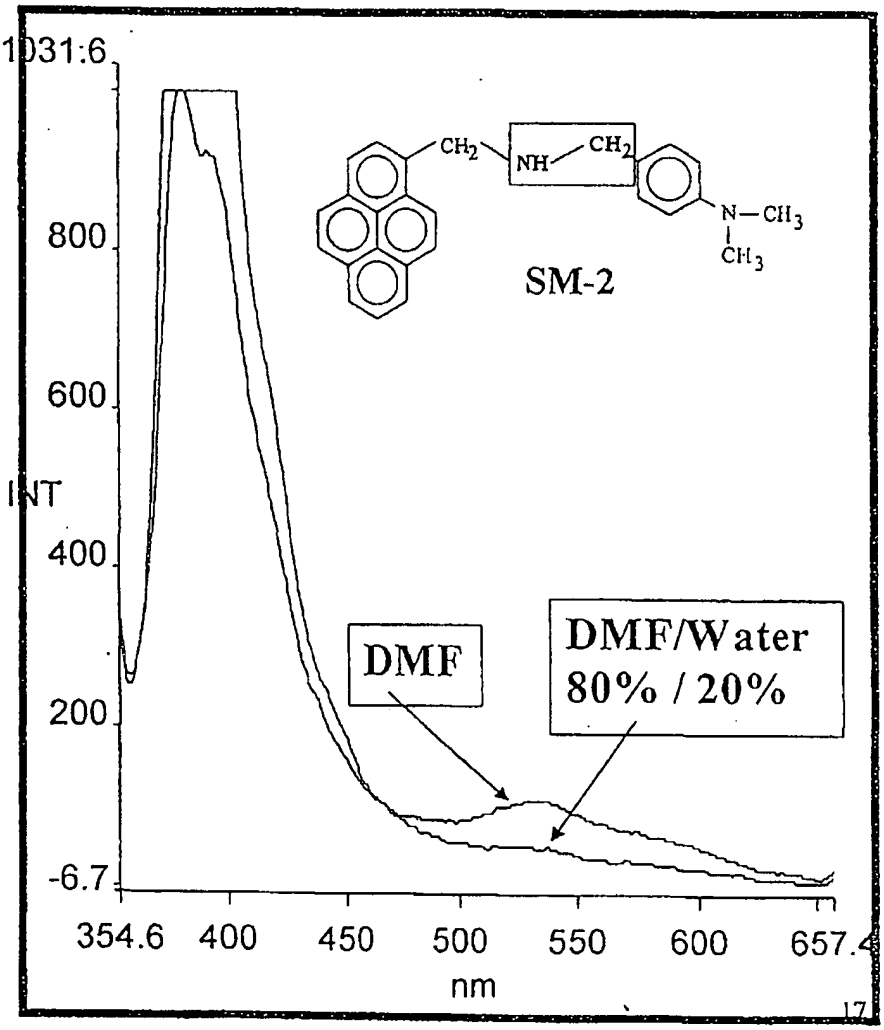


FIG.3

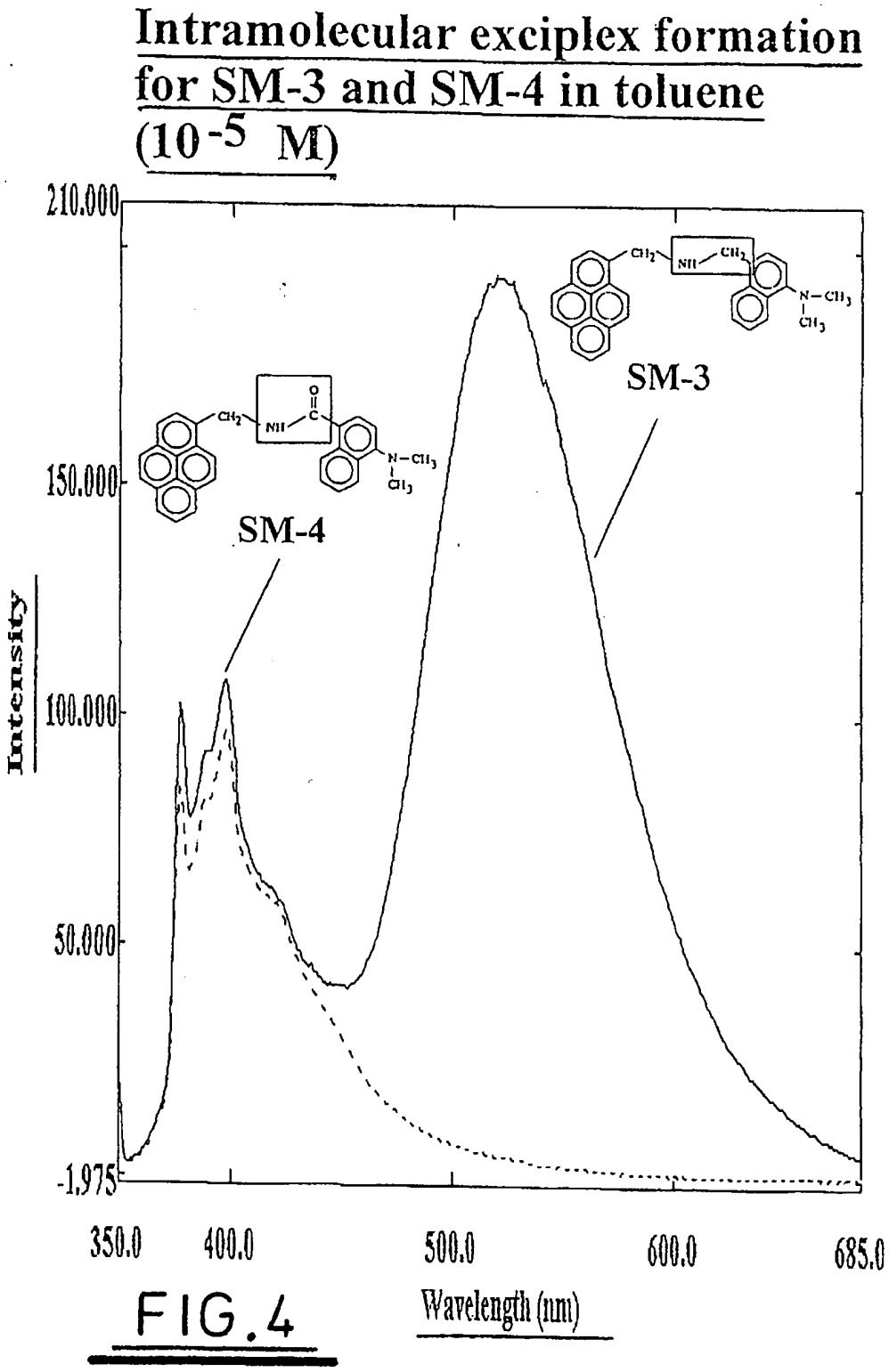

Intramolecular exciplex formation for SM-3 and SM-4 in toluene (10^{-5} M)
210.000
150.000
100.000
50.000
-1.975
Intensity
SM-3
SM-4
350.0
400.0
500.0
600.0
685.0
Wavelength (nm)

FIG. 4

Intramolecular exciplex formation for SM-4 (-----) and SM-3 (——) in THF

(10^{-5} M)

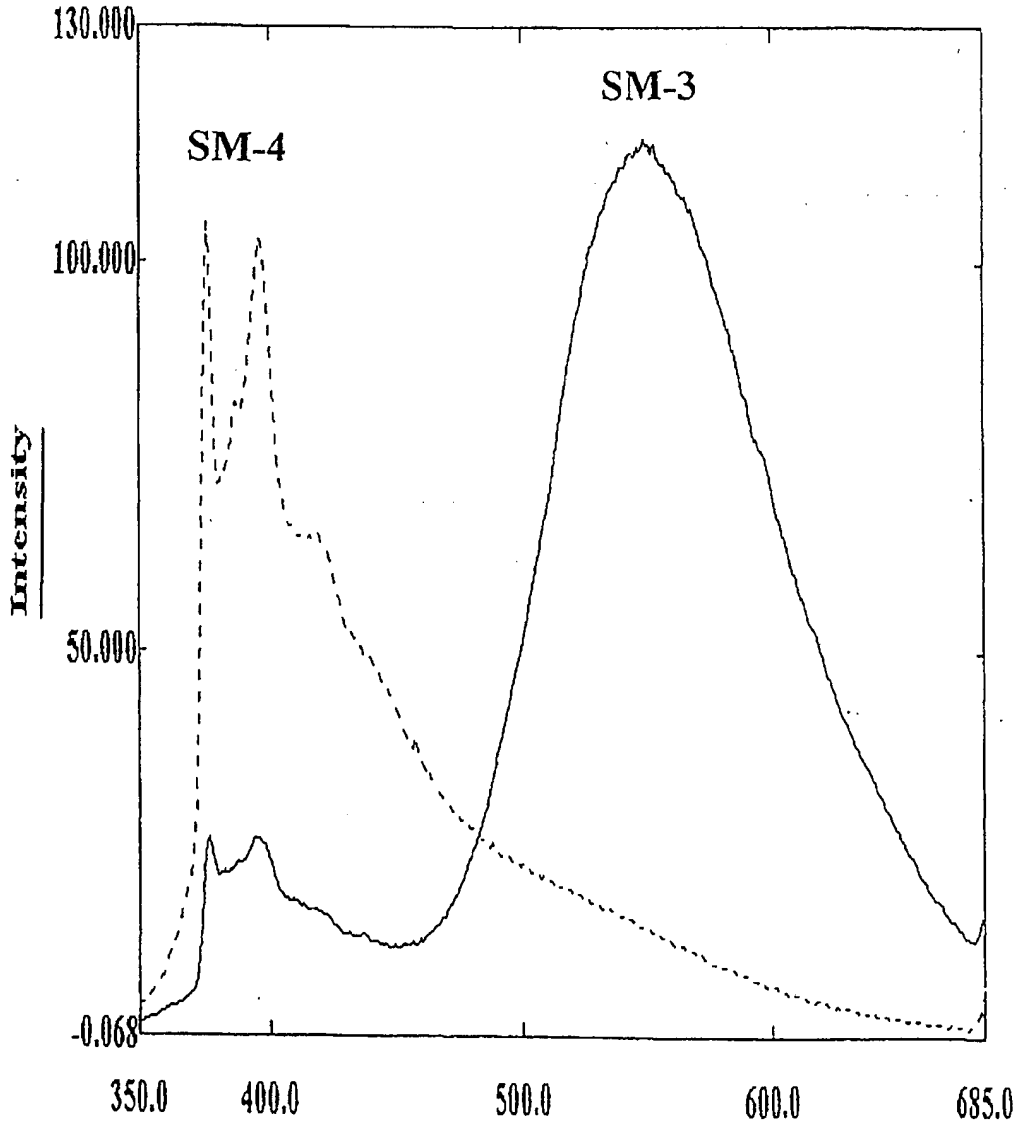


FIG. 5

Intramolecular exciplex formation for SM-1 (-----) and SM-2 (——) in Toluene

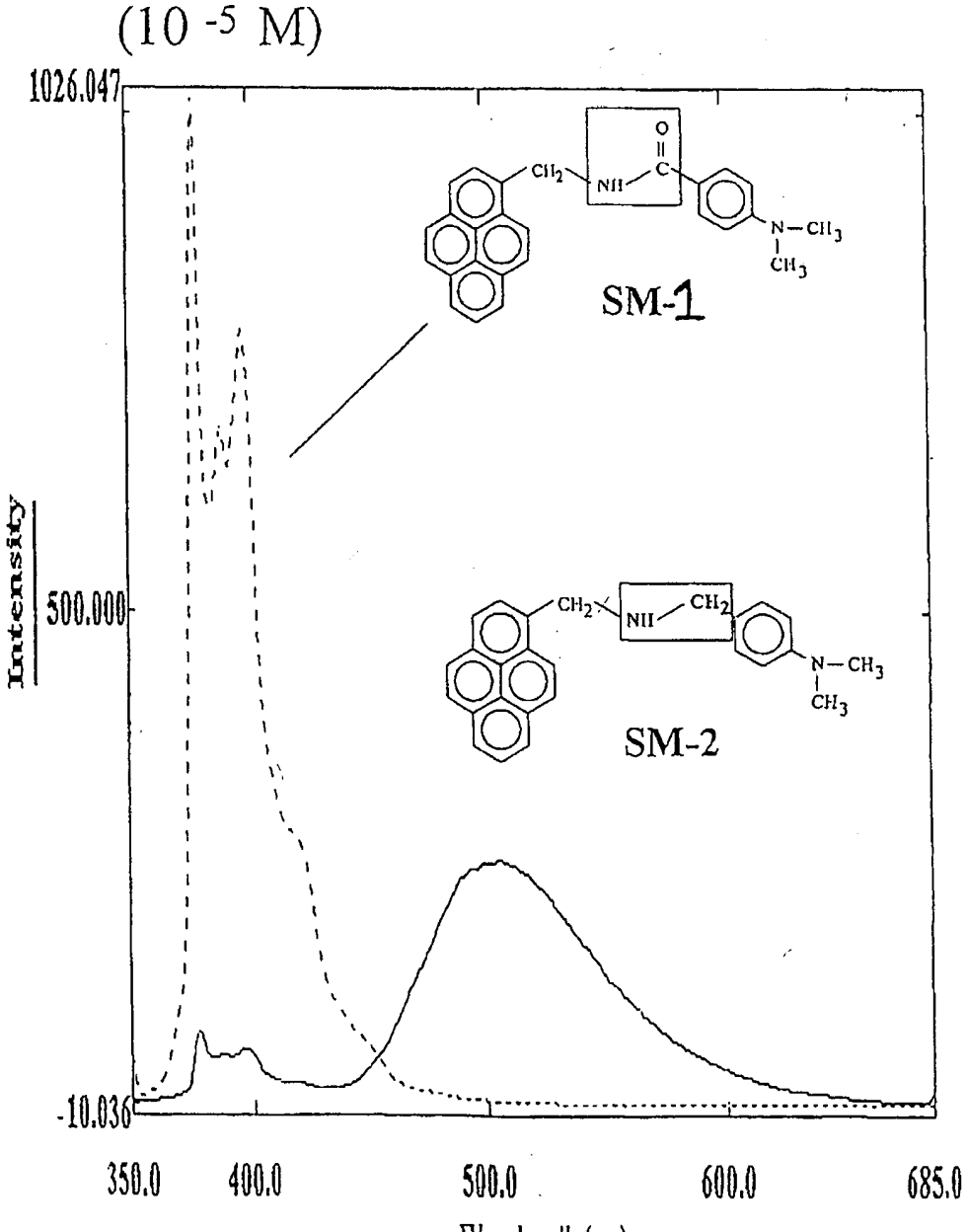


FIG. 6

Emission Spectra of SM-2 and Pyrenemethylamine in 10 mM Tris, pH9

(Excitation wavelength - 350 nm)

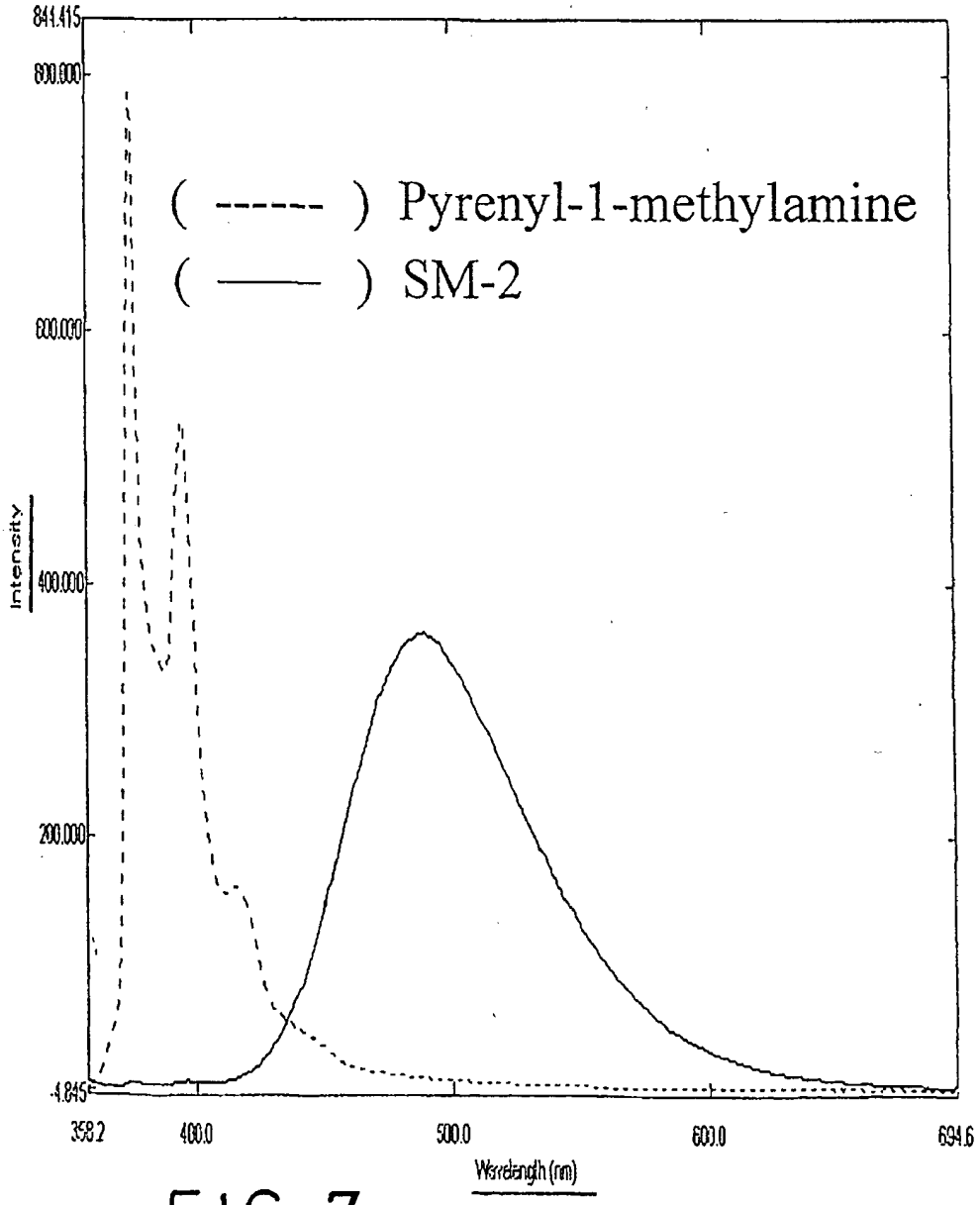


FIG. 7

Salt Infuence of Eciplex Emission for SM-2 (10, mM Tris, pH 9.0)

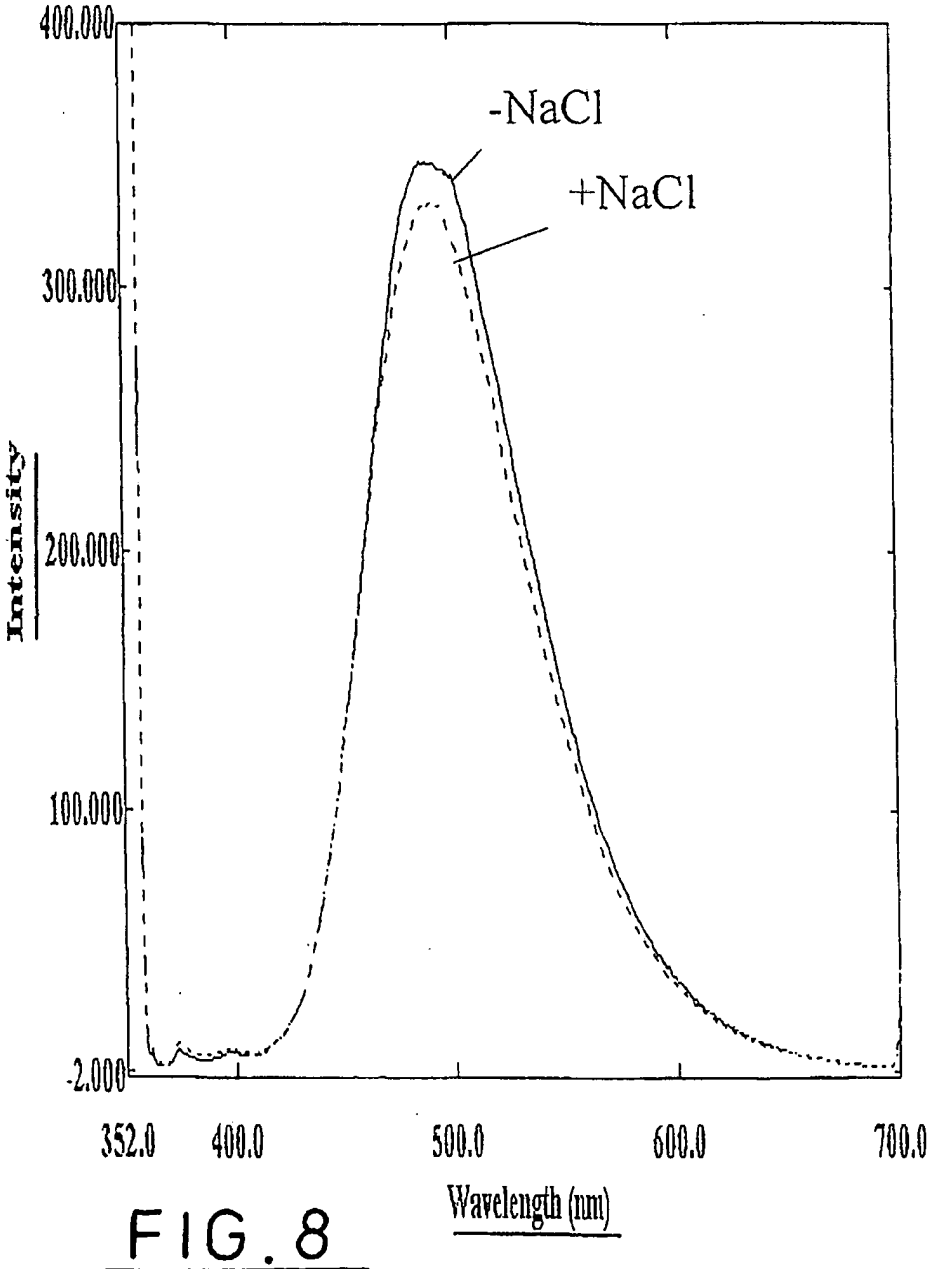


FIG. 8

pH-Dependence of Exciplex Emission for SM-3 (10 mM Tris bufffer, λ ext = 342 nm)

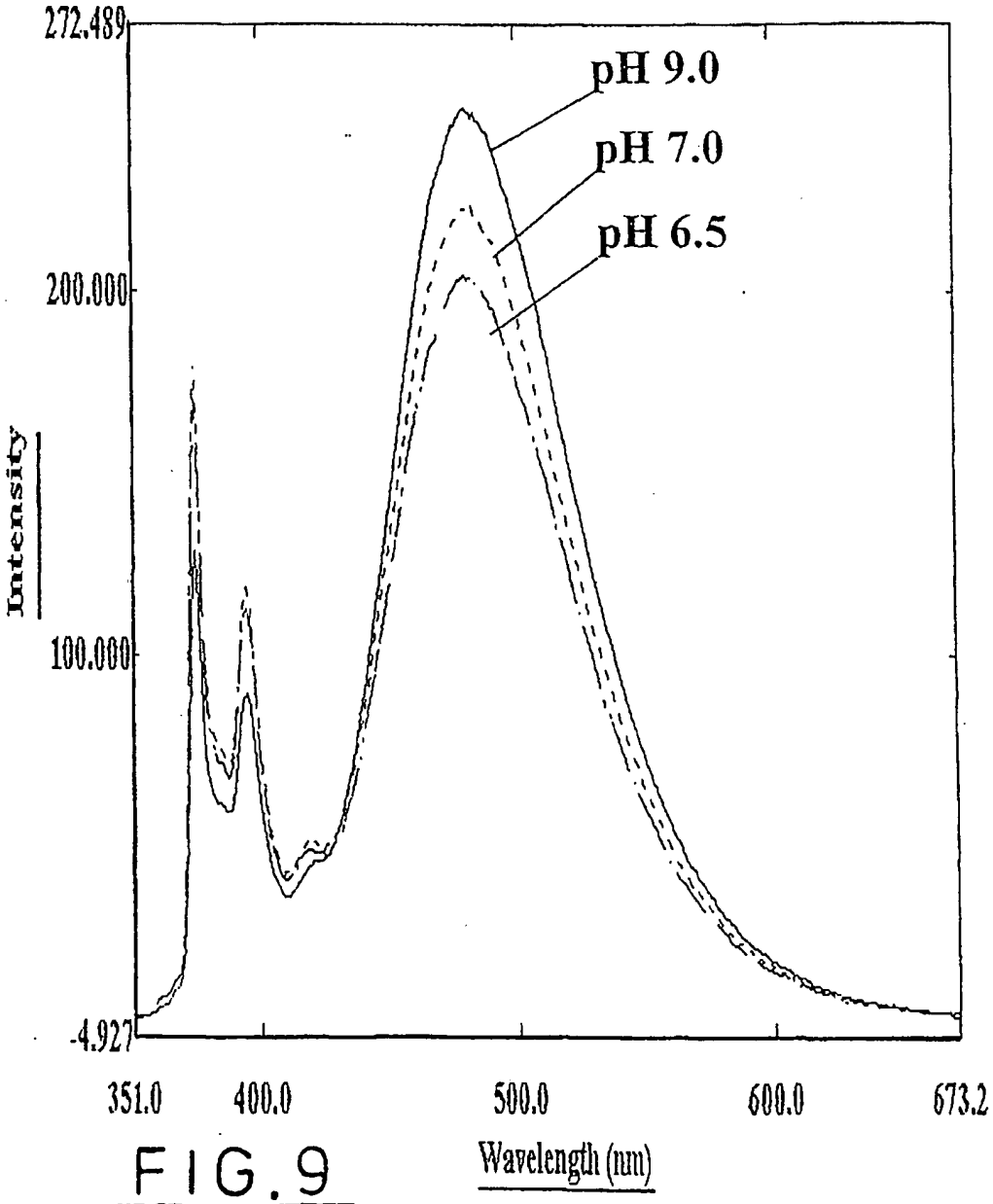


FIG. 9

Emission Spectra of SM-8 in different solvents

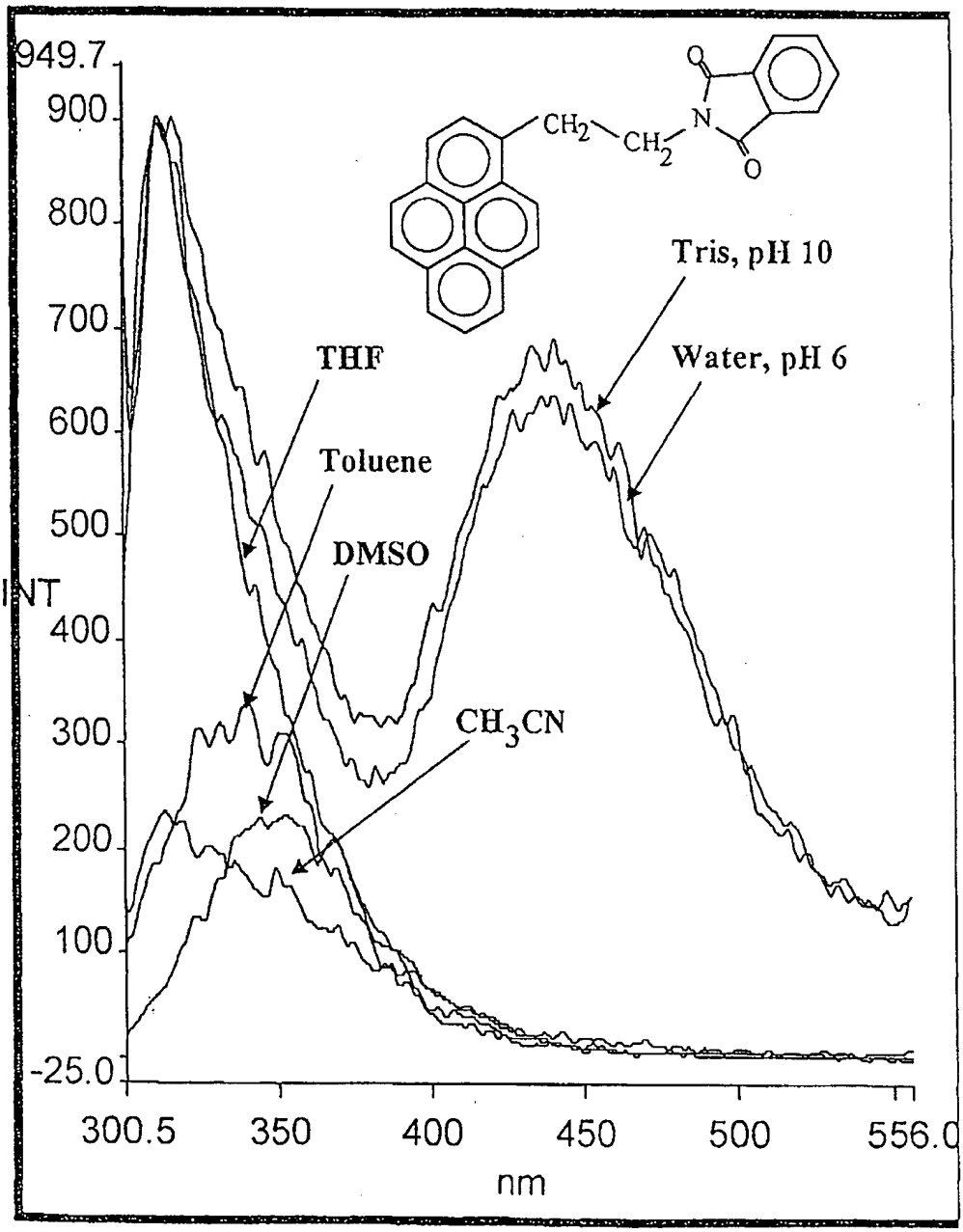


FIG. 10

Pyrene-DMA in H_2O with β-cyclodextrin

Concentration: (10^{-5}M)

(i) – Pyrene-CH_2-NH-CH_2-DMA in Tris buffer, pH 10.0 (without β-Cyclodextrin

(ii) – Pyrene-CH_2-NH-CH_2-DMA in H_2O + β-Cyclodextrine (pH 10.0)

(iii) – Pyrene-CH_2-NH-CH_2-DMA in H_2O + β-Cyclodextrin (pH 7.0)

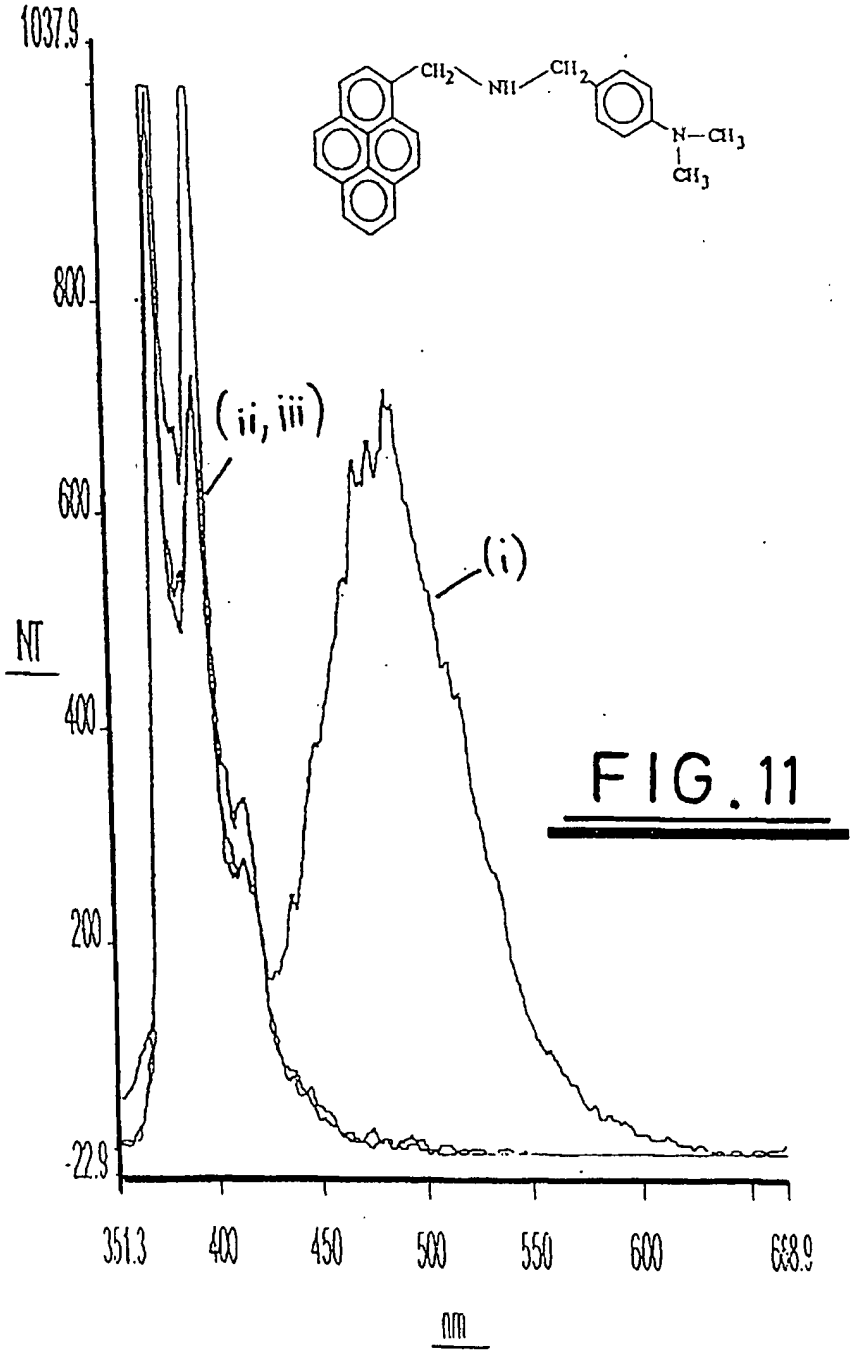


FIG. 11

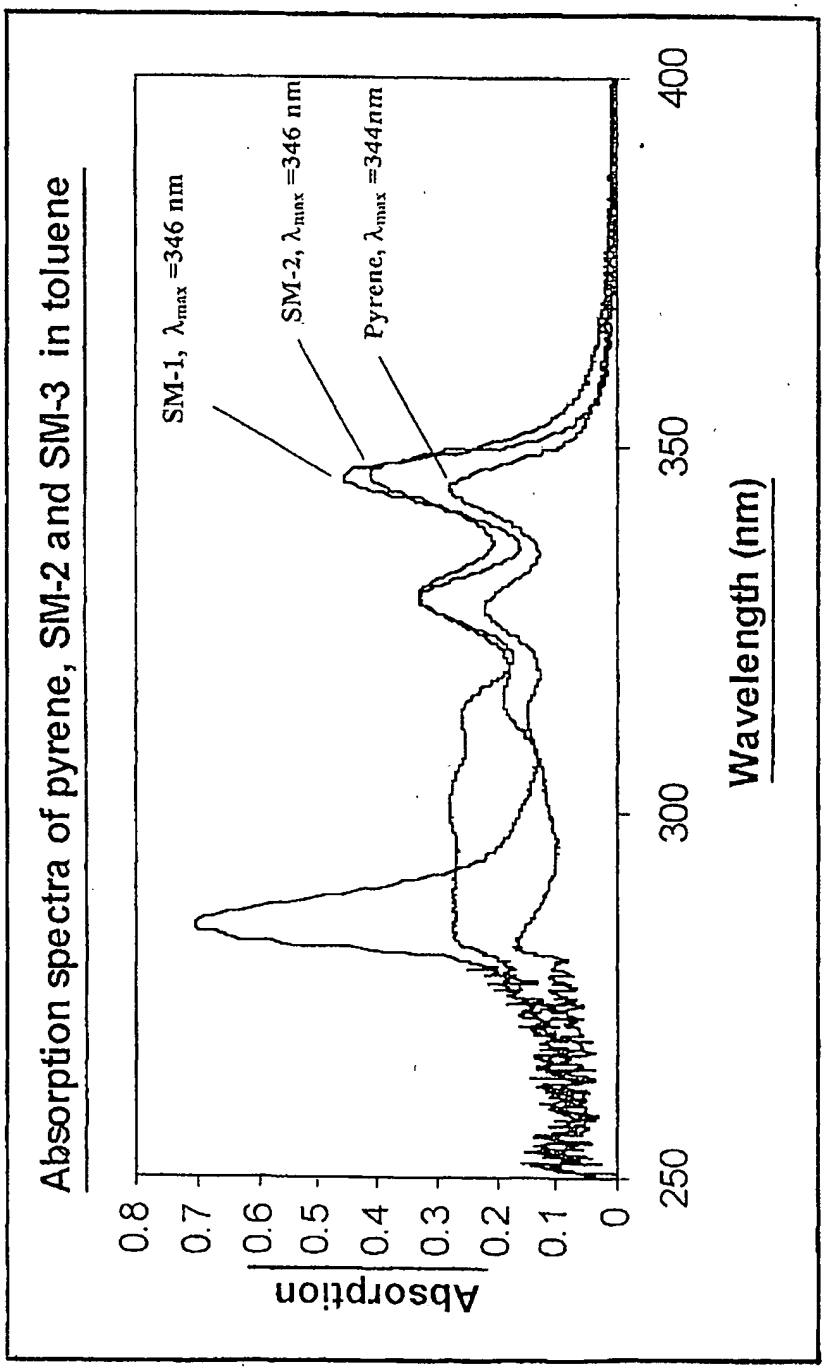

Absorption spectra of pyrene, SM-2 and SM-3 in toluene
SM-1, λ_{max} =346 nm
SM-2, λ_{max} =346 nm
Pyrene, λ_{max} =344nm
Absorption
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
250
300
350
400
Wavelength (nm)

FIG. 12

Excitation Spectra of SM-2 in Toluene

(———) for emission at 504 nm;

(------) for emission at 393 nm;*

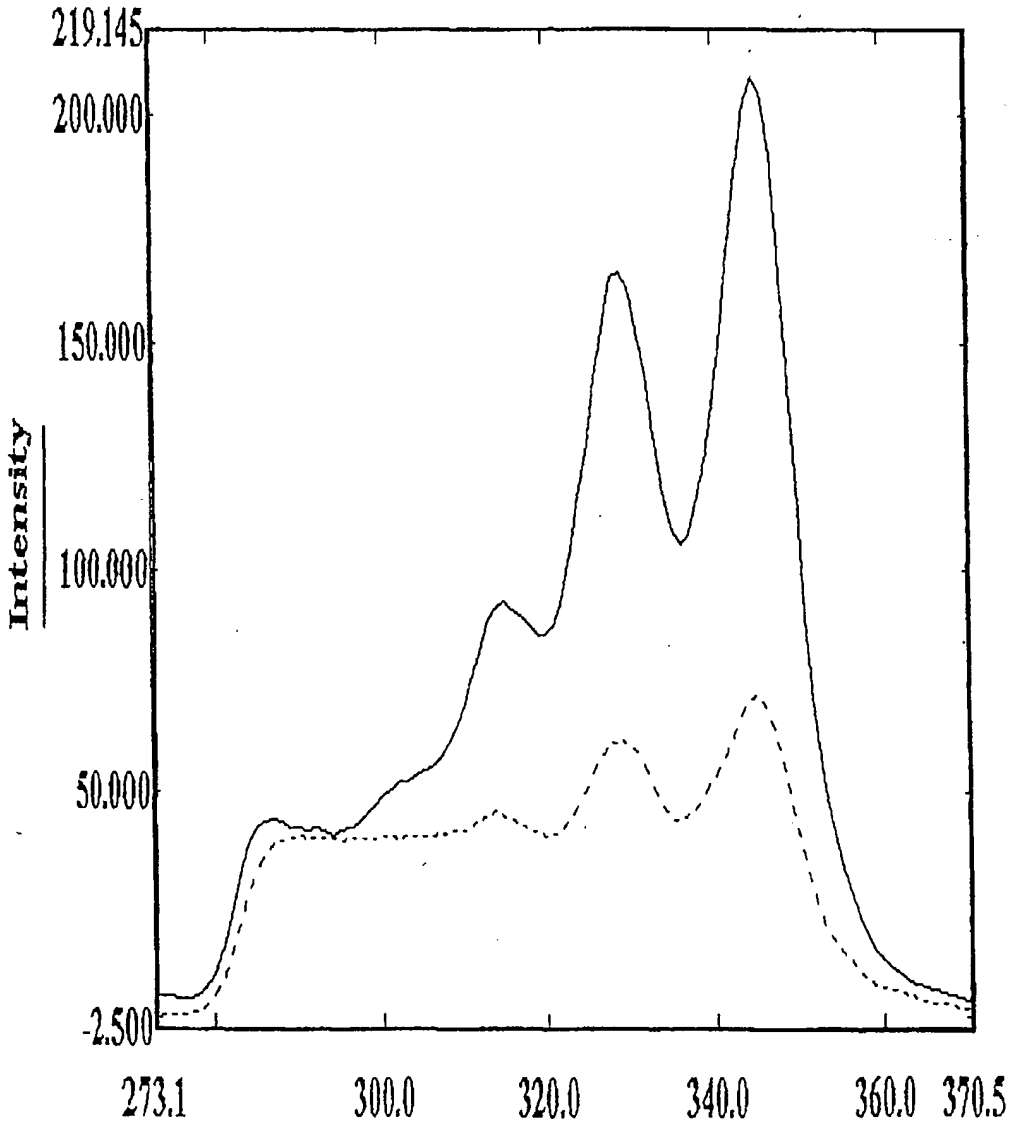


FIG. 13

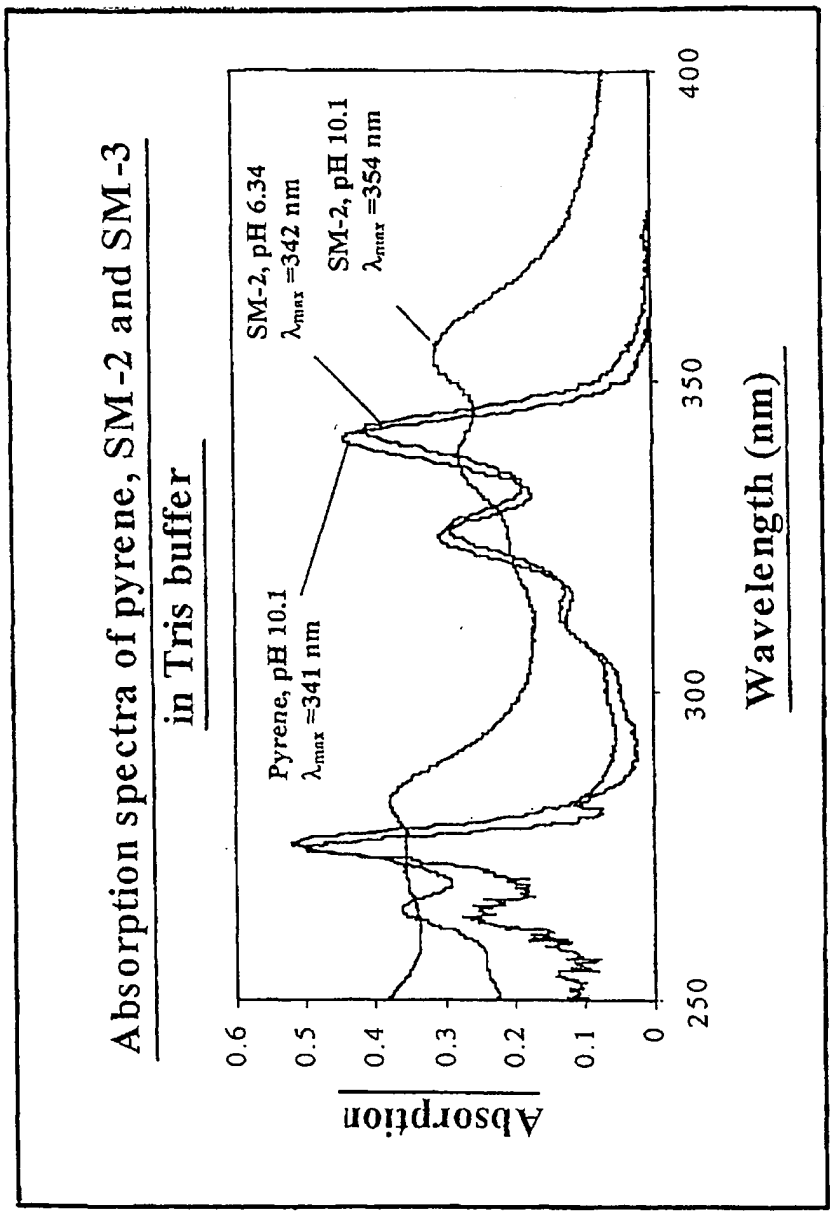

Absorption spectra of pyrene, SM-2 and SM-3
in Tris buffer
Pyrene, pH 10.1
λmax =341 nm
SM-2, pH 6.34
λmax =342 nm
SM-2, pH 10.1
λmax =354 nm
Absorption
0.6
0.5
0.4
0.3
0.2
0.1
0
250
300
350
400
Wavelength (nm)

FIG.14

Excitation Spectra of SM-2 in 10 mm Tris, pH 9.0

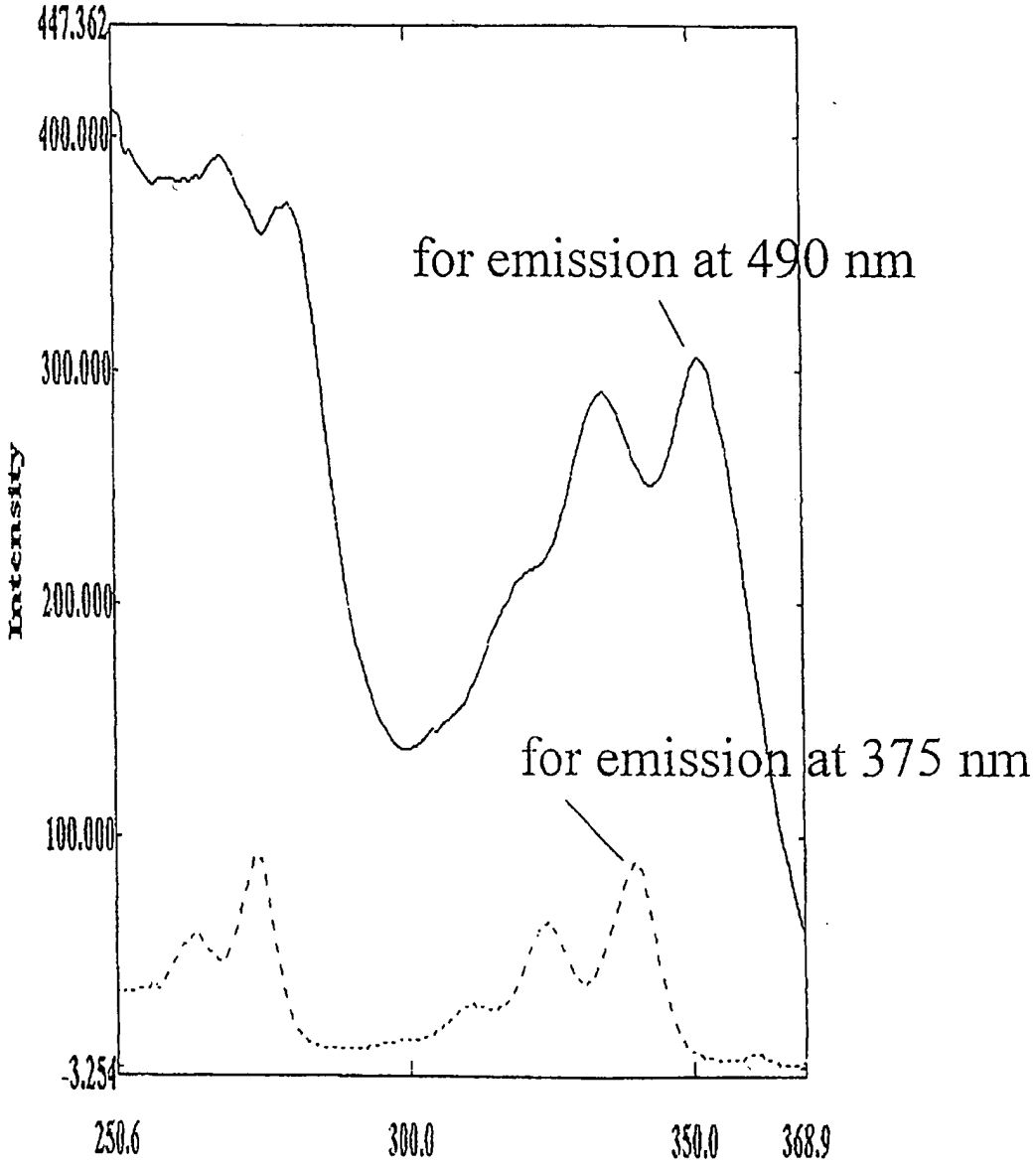


FIG.15

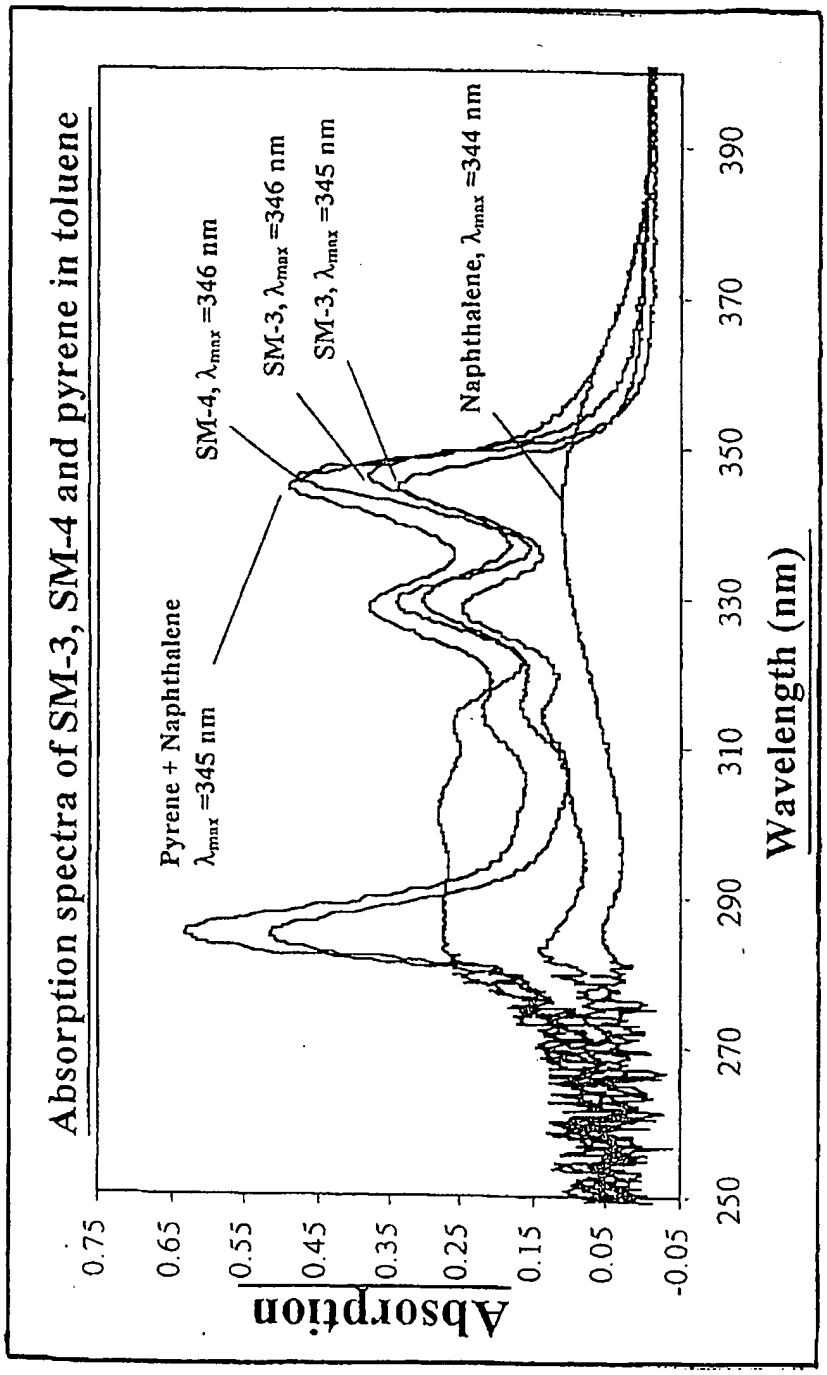

Absorption spectra of SM-3, SM-4 and pyrene in toluene
Pyrene + Naphthalene
λ_{max} =345 nm
SM-4, λ_{max} =346 nm
SM-3, λ_{max} =346 nm
SM-3, λ_{max} =345 nm
Naphthalene, λ_{max} =344 nm
Absorption
0.75
0.65
0.55
0.45
0.35
0.25
0.15
0.05
-0.05
250
270
290
310
330
350
370
390
Wavelength (nm)

FIG. 16

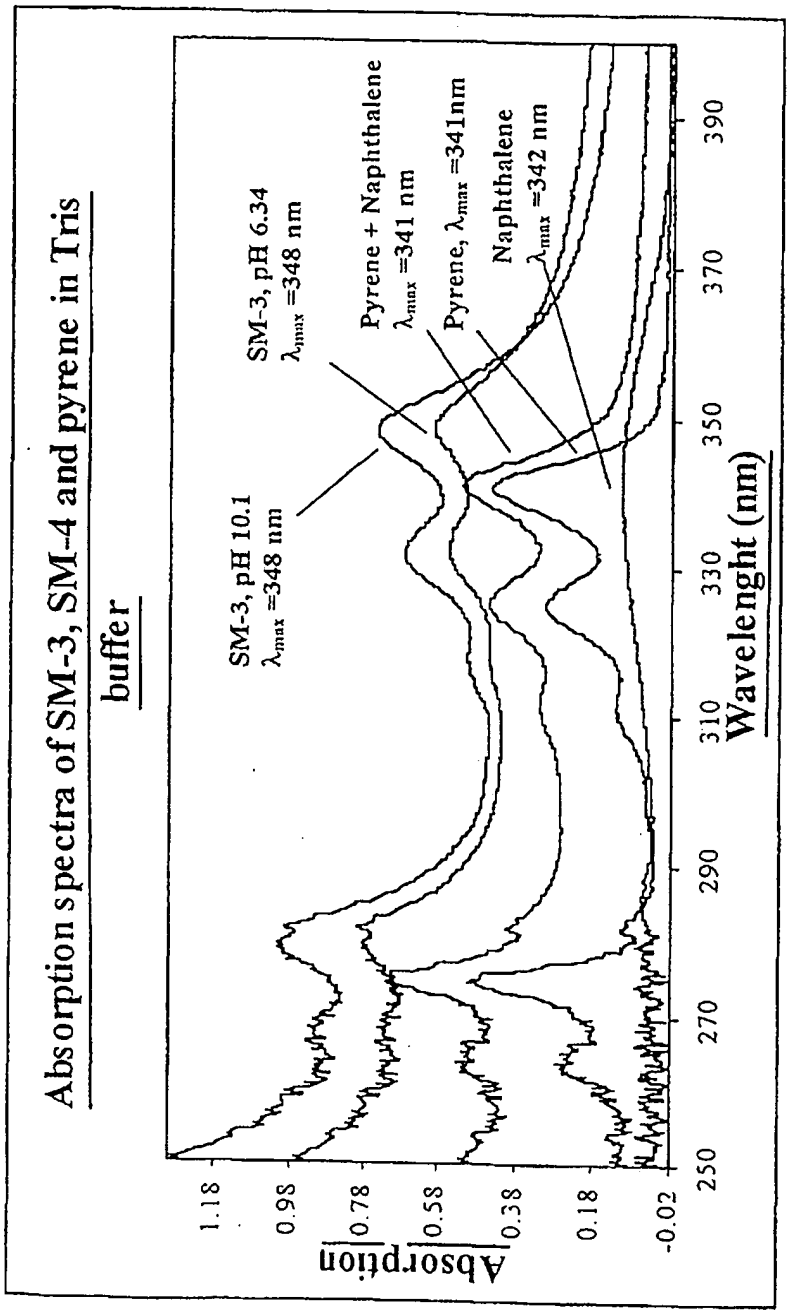

Absorption spectra of SM-3, SM-4 and pyrene in Tris buffer
SM-3, pH 10.1
λmax =348 nm
SM-3, pH 6.34
λmax =348 nm
Pyrene + Naphthalene
λmax =341 nm
Pyrene, λmax =341nm
Naphthalene
λmax =342 nm
Absorption
1.18
0.98
0.78
0.58
0.38
0.18
-0.02
250
270
290
310
330
350
370
390
Wavelenght (nm)

FIG.17

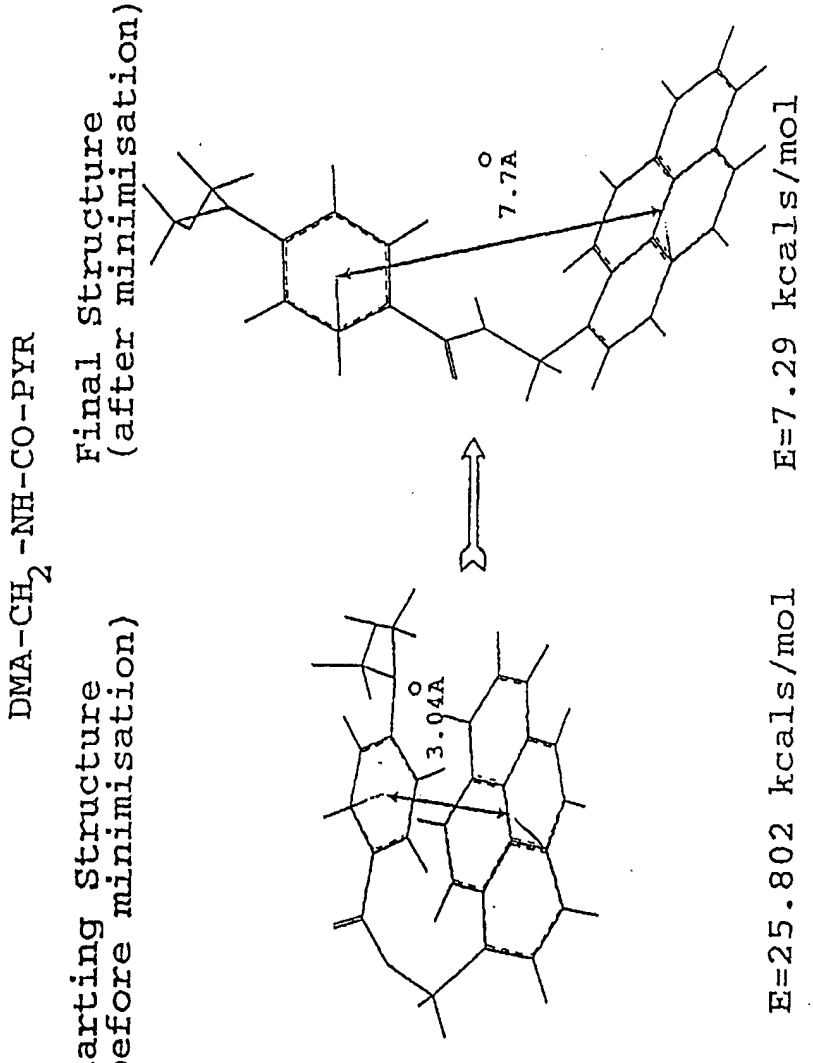

DMA-CH2-NH-CO-PYR
Starting Structure
(before minimisation)
Final Structure
(after minimisation)
3.94A
7.7A
E=25.802 kcals/mol
E=7.29 kcals/mol

FIG.18

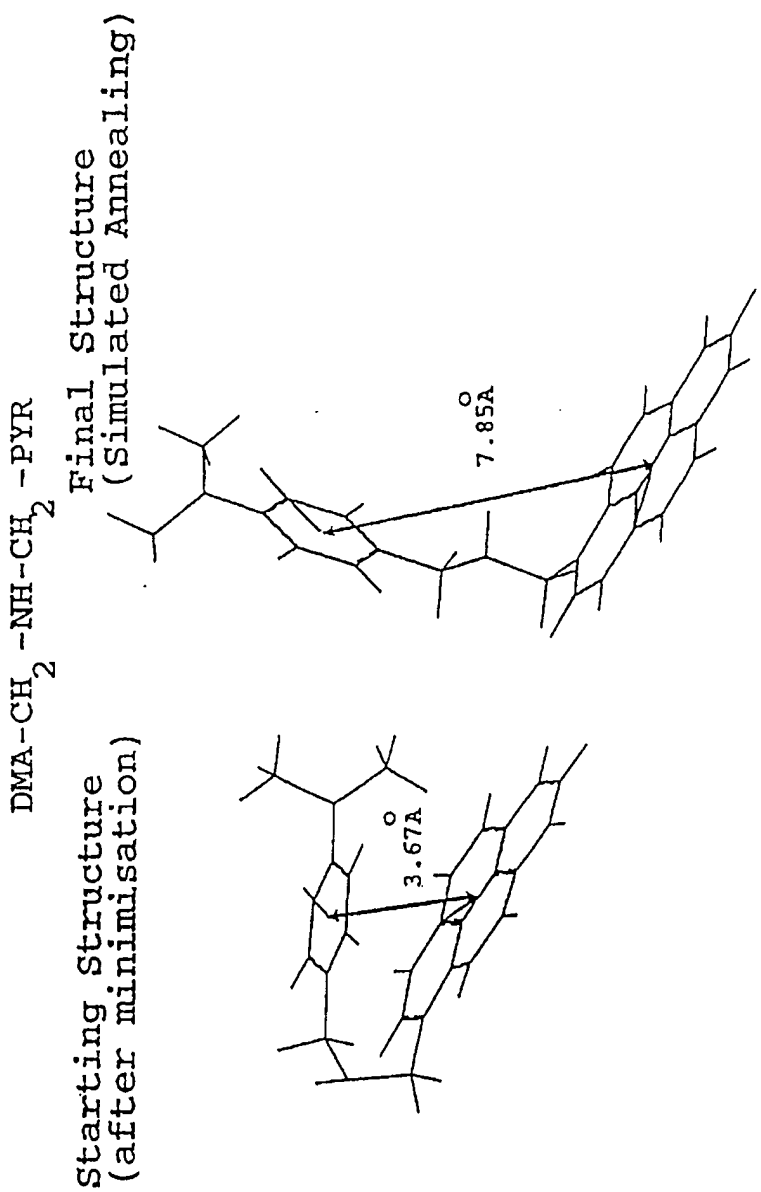
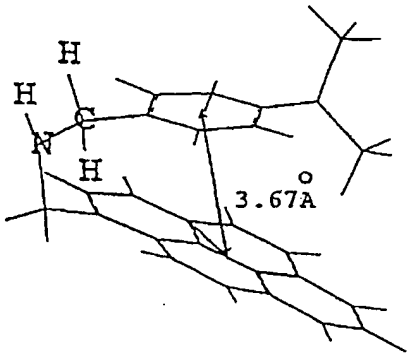

DMA-CH2-NH-CH2-PYR
Starting Structure
(after minimisation)
3.67Å
E = 1.689 kcals/mol
Final Structure
(Simulated Annealing)
7.85Å
E= 6.029 kcals/mol

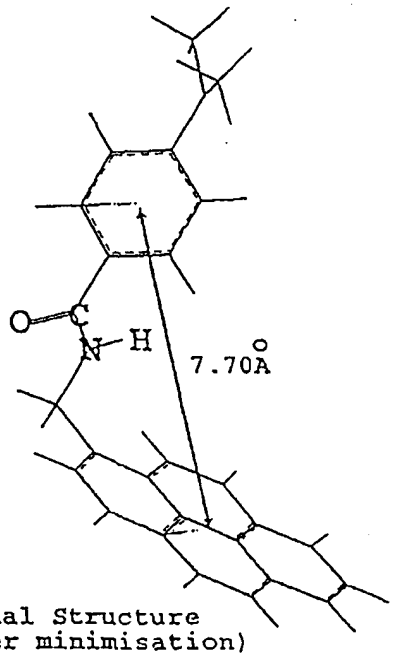
FIG.19

DMA-CH_2-NH-CH_2-PYR



Final Structure
(after minimisation)

DMA-CH_2-NH-CO-PYR



Final Structure
(after minimisation)

FIG. 20

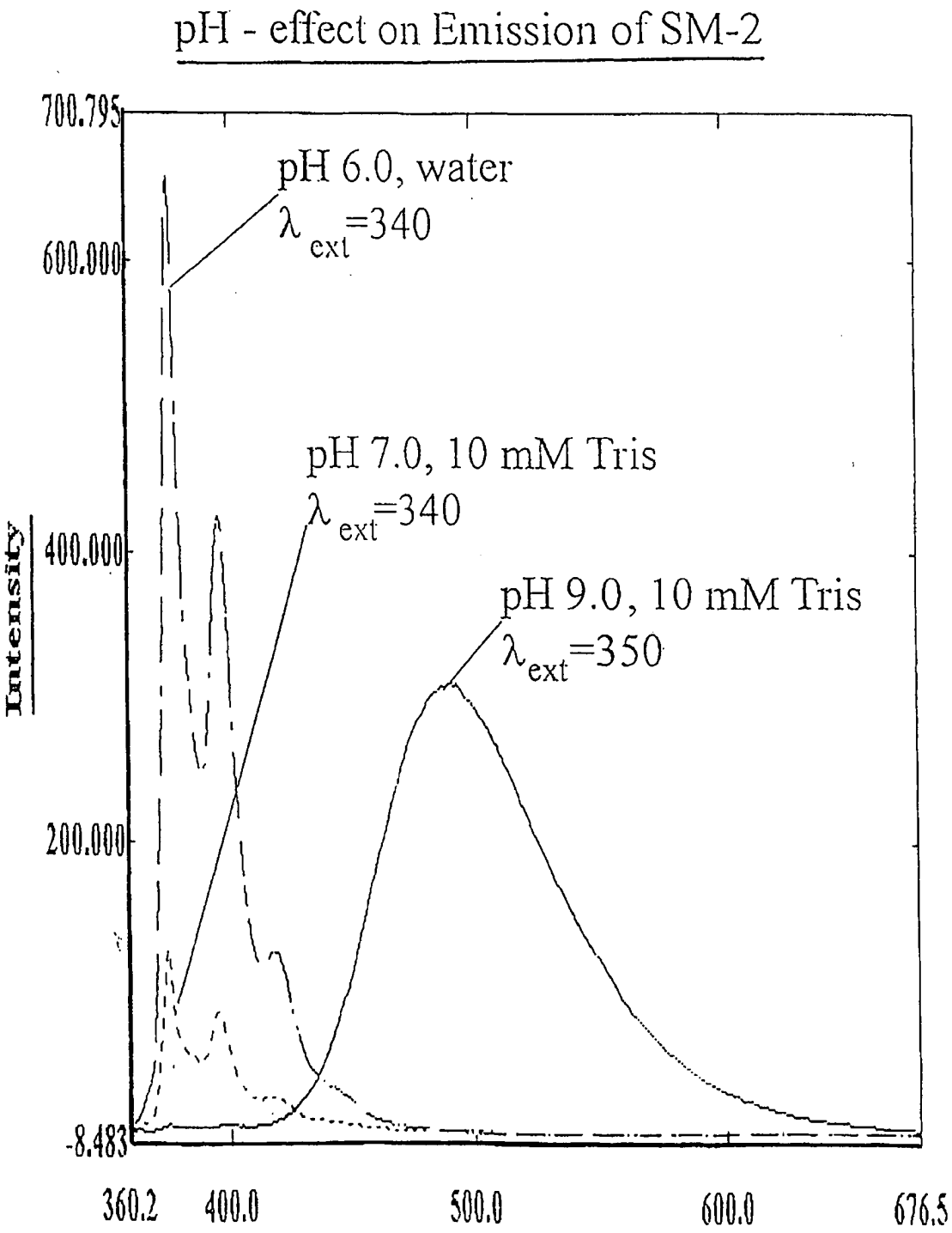
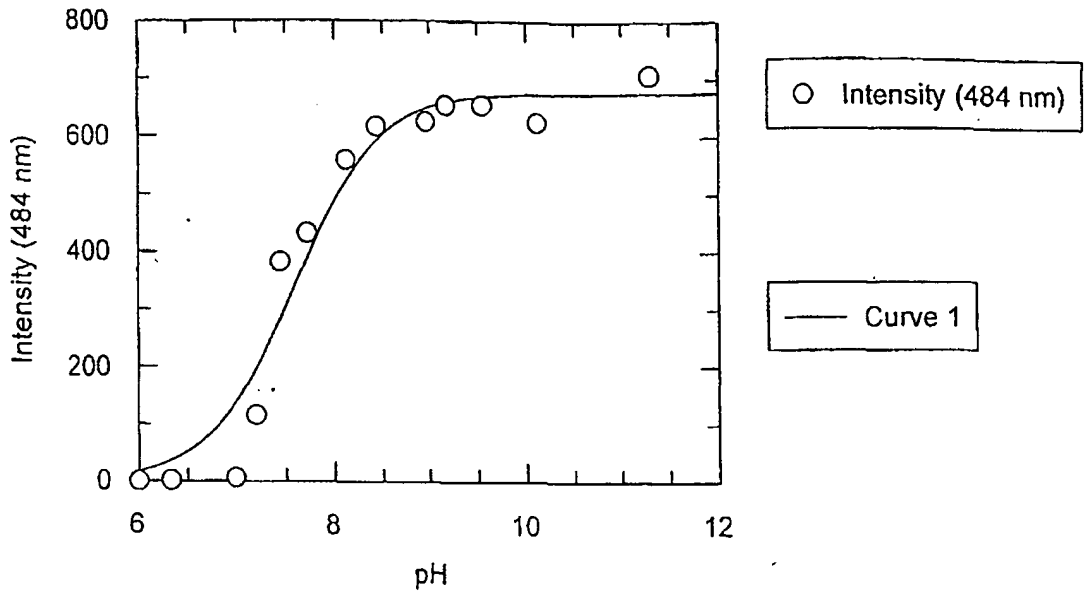

pH - effect on Emission of SM-2
700.795
600.000
400.000
200.000
-8.483
Intensity
pH 6.0, water
λ ext=340
pH 7.0, 10 mM Tris
λ ext=340
pH 9.0, 10 mM Tris
λext=350
360.2
400.0
500.0
600.0
676.5

FIG. 21



pKa Determination,
minimum = 0
Simple weighting
Reduced Chi squared =
3877

Variable	Value	Std. Err.
pKa	7.5812	0.0957
Limit	676.4241	27.5185

FIG. 21a

Pyrene-DMA in H_2O at different pH;

Concentration: (10^{-4}M)
(xi) – Pyrene-CH_2-NH-CH_2-DMA in H_2O (pH 6.0);
(xv) – Pyrene-CH_2-NH-CH_2-DMA in Tris buffer (pH 10.0);
(xiii) – Pyrene-CH_2-NH-CH_2-DMA in H_2O + NaOH (pH~14);
(xii) – Pyrene-CH_2-NH-CH_2-DMA in Na-phosphate buffer (pH 7.0);
(xiv) – Pyrene-CH_2-NH-CH_2-DMA in NaH_2PO_4/ Na_2HPO_4buffer + NaOH (pH ~14);

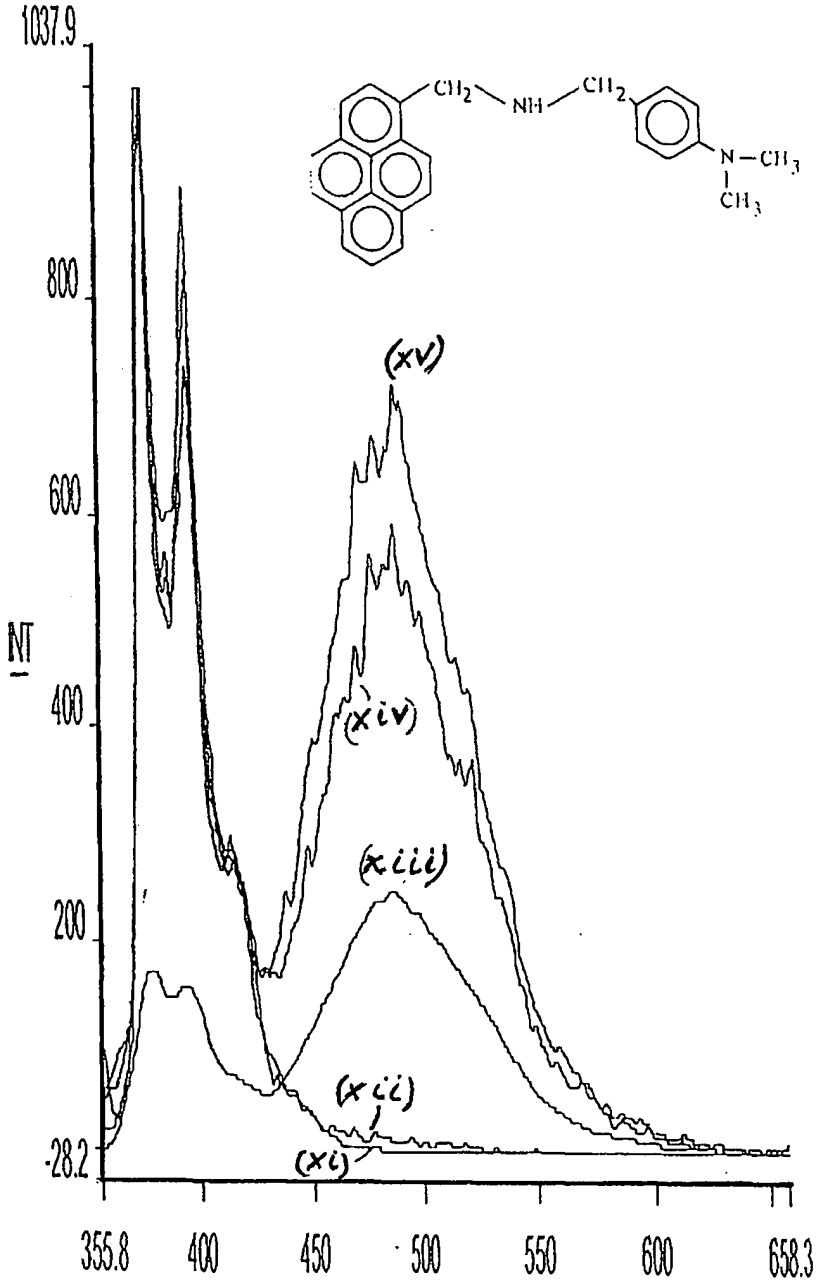


FIG. 22

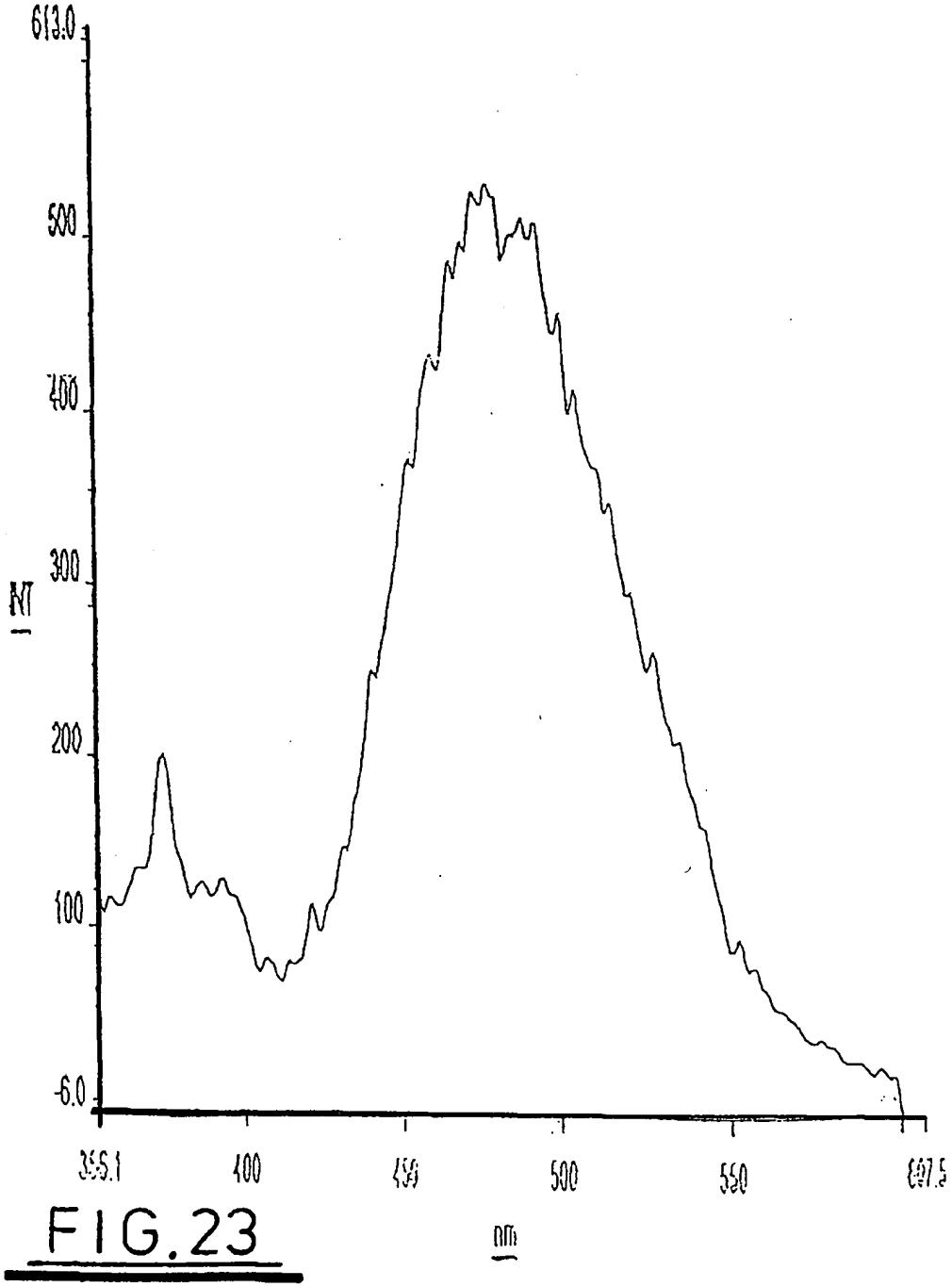


FIG.23

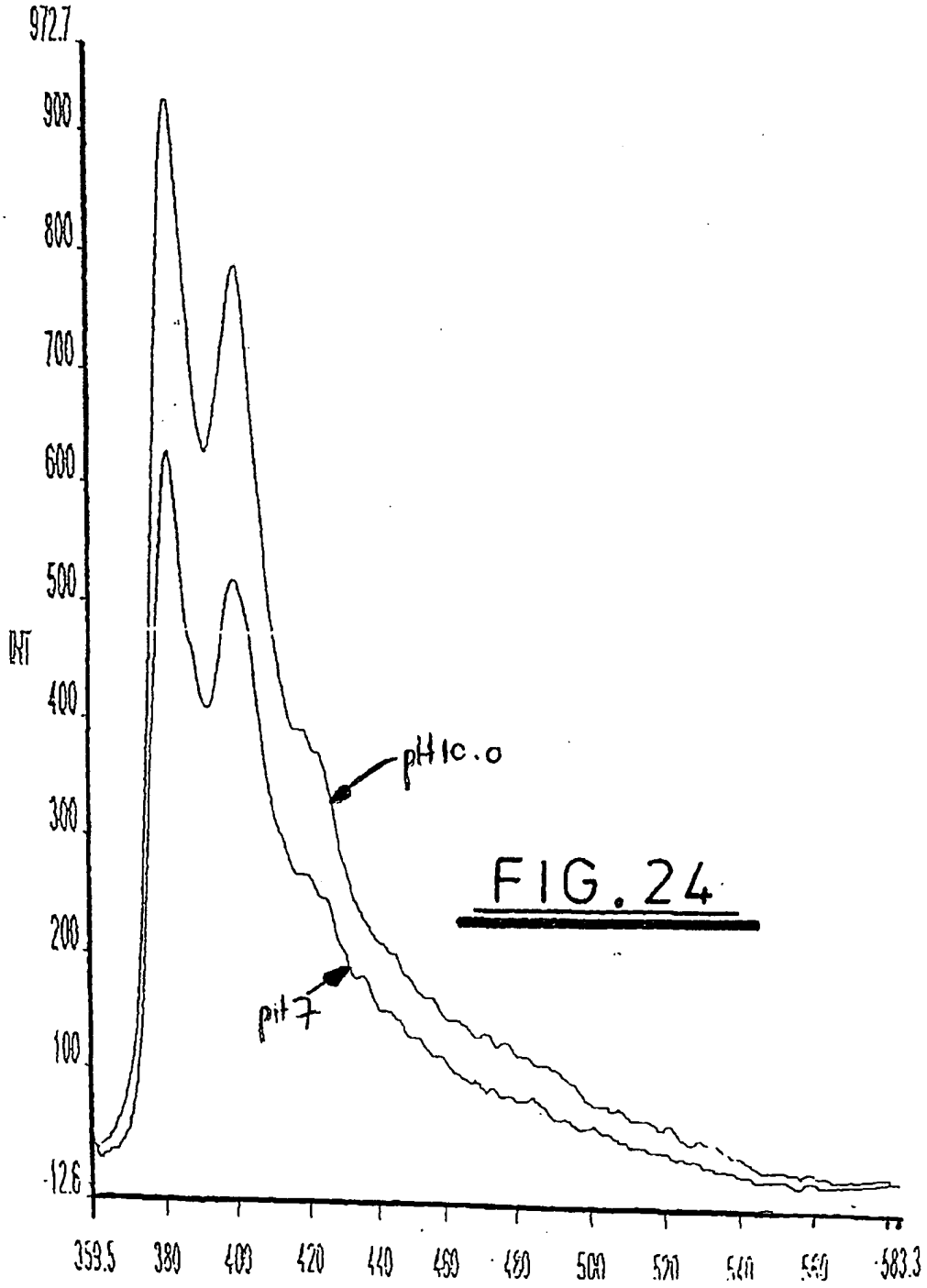

972.7
900
800
700
600
500
400
300
200
100
-12.6
INT
pH 1c.o
pH 7
359.5
380
400
420
440
460
480
500
520
540
560
583.3

FIG. 24

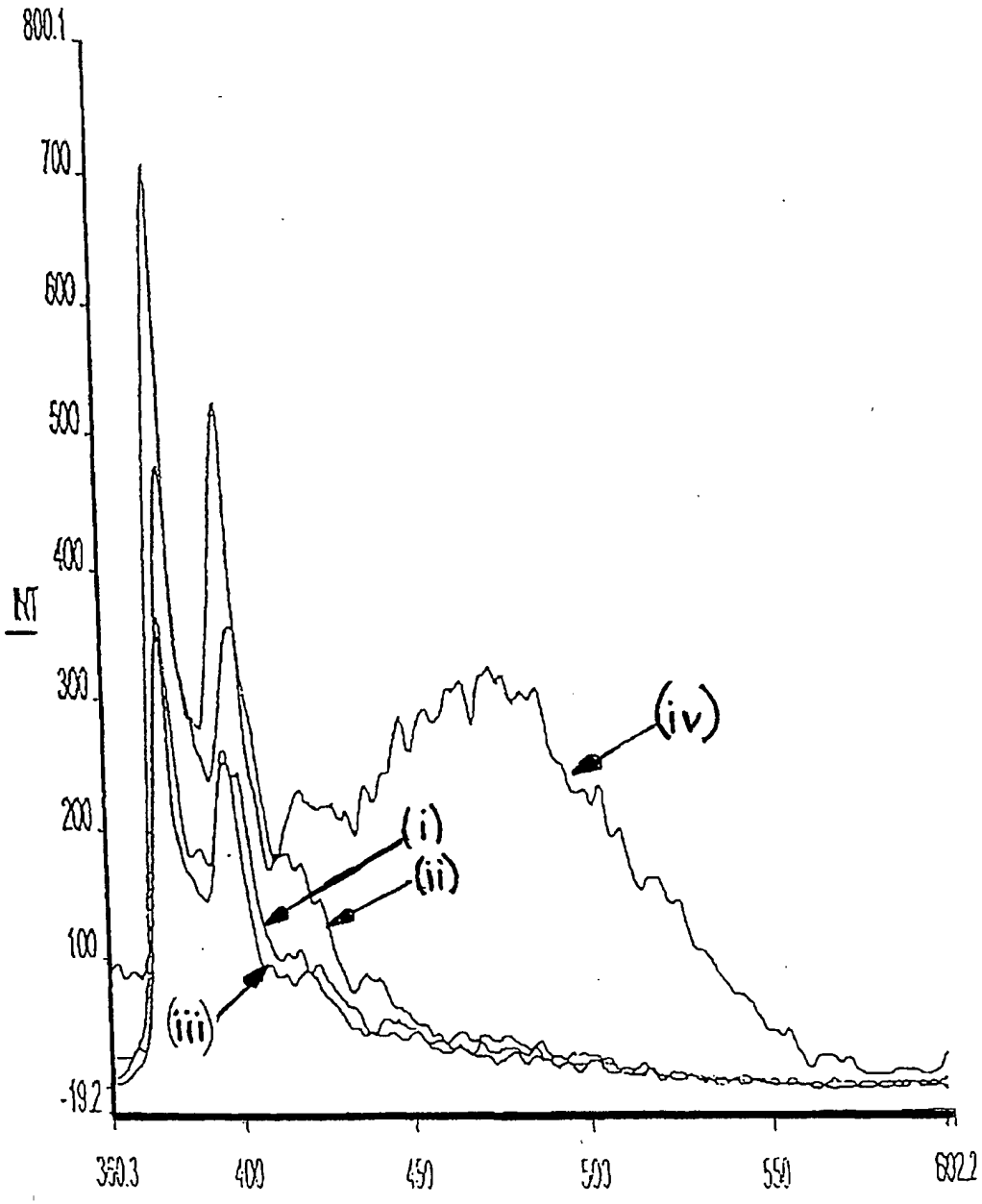

800.1
700
600
500
400
300
200
100
-19.2
INT
(i)
(ii)
(iii)
(iv)
350.3
400
450
500
550
802.2
INT

FIG. 25

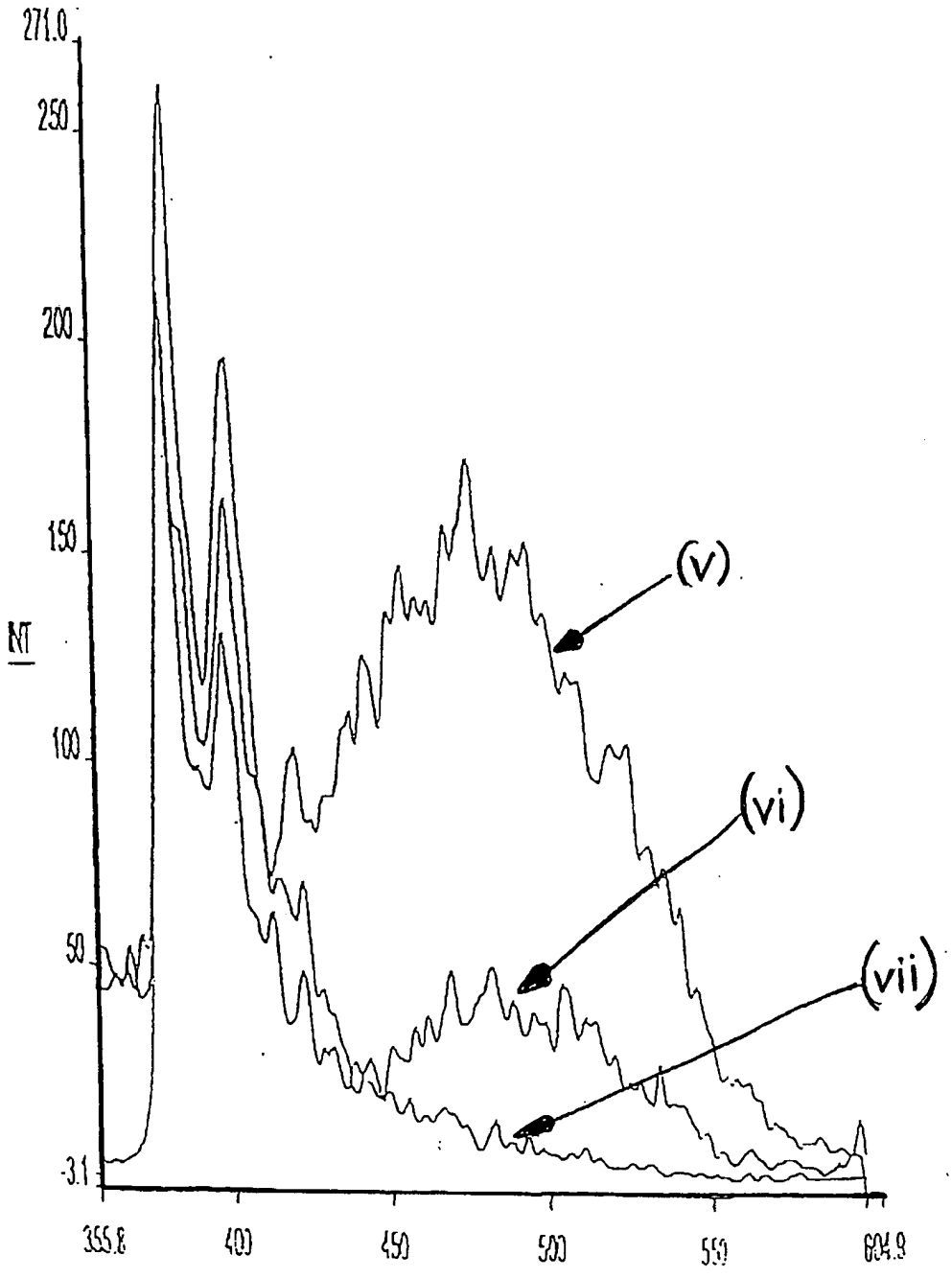

271.0
250
200
150
100
50
-3.1
NT
355.8
400
450
500
550
604.9
nm
(v)
(vi)
(vii)

FIG. 26

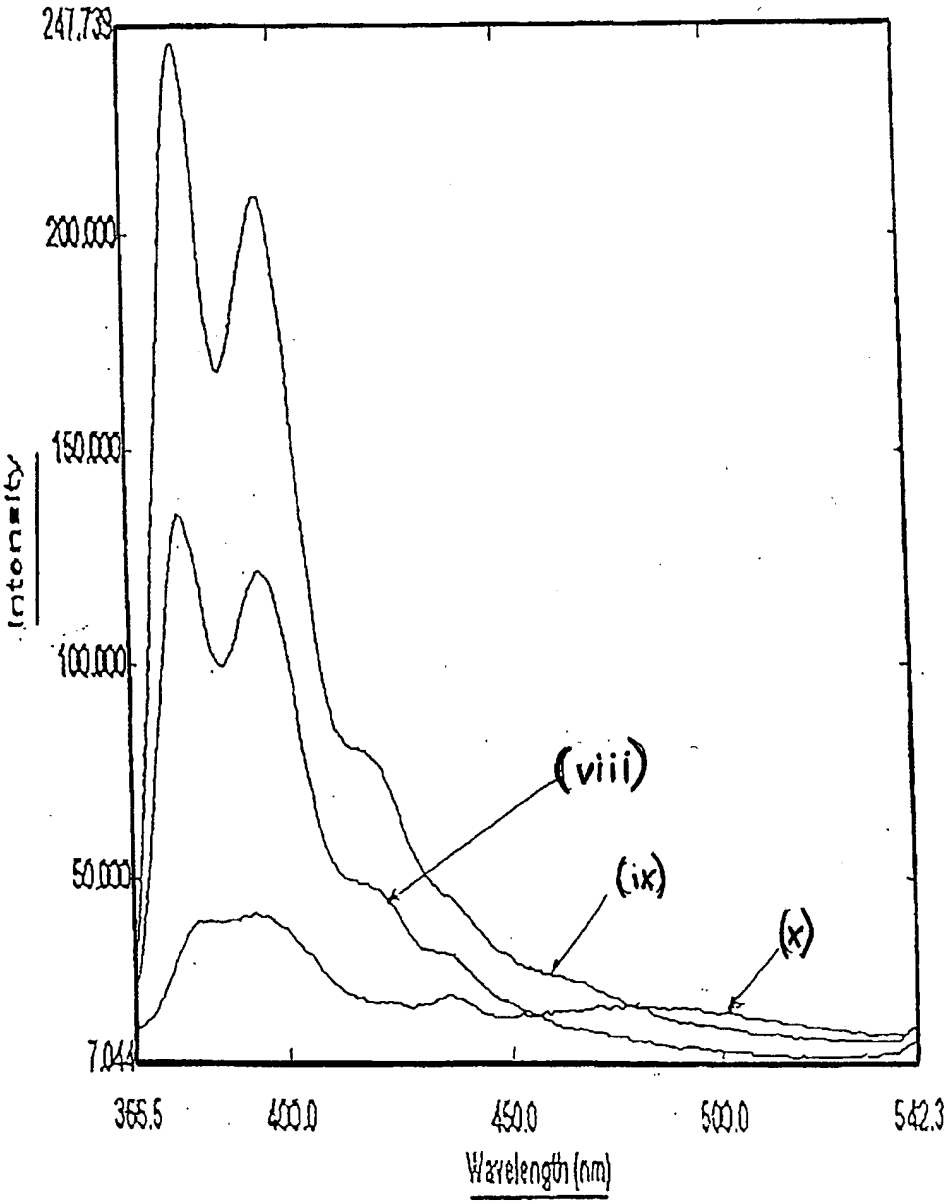

247.739
200.000
150.000
100.000
50.000
7.044
Intensity
(viii)
(ix)
(x)
365.5
400.0
450.0
500.0
542.3
Wavelength (nm)

FIG. 27

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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International Bureau

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(26) Publication Language: English

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(71) Applicant *(for all designated States except US)*: **THE VICTORIA UNIVERSITY OF MANCHESTER** [GB/GB]; Oxford Road, Manchester M13 9PL (GB).

(72) Inventors; and

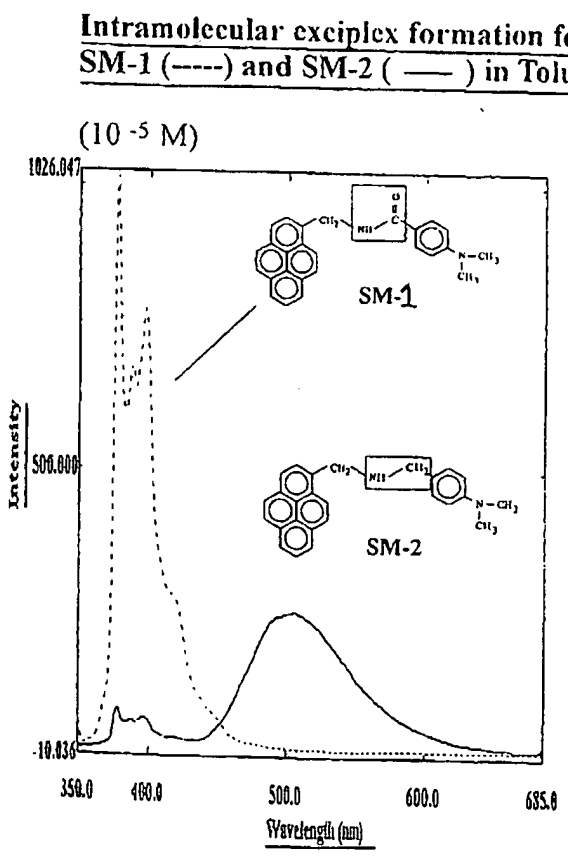
(75) Inventors/Applicants *(for US only)*: **DOUGLAS, Kenneth, Thomas** [GB/GB]; Higher Shore Cottage, Higher Shore Road, Littleborough OL15 9LW (GB). **BICHENKOVA, Elena, Vladimirovna** [LV/GB]; 2 Egerton Court, Upper Park Road, Manchester M14 5SL (GB). **SARDARIAN, Ali** [IR/GB]; Flat 210, Horniman House, 66 Grafton Street, Manchester M13 9NT (GB).

(74) Agent: **ATKINSON, Peter, Birch**; Marks & Clerk, Sussex House, 83-85 Mosley Street, Manchester M2 3LG (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

[Continued on next page]

(54) Title: EXCIPLEXES



(57) Abstract: Compounds capable of forming an intramolecular exciplex on photoirradiation of the compound in water comprise two exciplex forming partners, one being a donor moiety and the other an acceptor moiety, each having at least one aromatic nucleus and being connected by a saturated aliphatic chain having the flexibility to allow said partners to come into exciplex forming relationship. The compounds may be used as labels for oligonucleotides. Certain of the compounds display pH sensitive emission.

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(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) **Date of publication of the international search report:**
20 December 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/04898

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07C211/49 C07D209/48 C07H19/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07C C07D C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 00 37674 A (BICHENKOVA ELENA VLADIMIROVNA ;UNIV MANCHESTER (GB); DOUGLAS KENNE) 29 June 2000 (2000-06-29) page 14 -page 18	1-30
A	US 5 466 578 A (KIDWELL DAVID A) 14 November 1995 (1995-11-14) column 1-3; figures 12A-13B	1-30

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2001	27/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sánchez García, J.M.

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/04898

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0037674	A	29-06-2000	AU	1867500 A	12-07-2000
			WO	0037674 A1	29-06-2000
US 5466578	A	14-11-1995	US	5332659 A	26-07-1994
			US	5314802 A	24-05-1994